Identification of a role for G protein-coupled receptor kinase 5 in regulating apoptosis

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Declaration

I, Katrina Nadine Lester, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in this thesis.

Signed…… Date……

Abstract

The G protein-coupled receptor kinases (GRKs) are best known for their role in phosphorylating G protein-coupled receptors (GPCRs). In addition to their canonical role, the GRKs have a number of non-receptor substrates and binding partners. GRK5 contains functional nuclear localisation and export sequences and many newly discovered binding partners of the kinase are, indeed, nuclear.

My thesis outlines the discovery of novel nuclear GRK5 binding partners, class I histone deacetylases (HDACs), HDACs 1, 3 and 8, as well as the repressor protein, Swi-independent 3 (Sin3) A, and reports a role for GRK5 in regulating B cell lymphoma protein 2 (Bcl-2) gene transcription in conjunction with this repressor complex. Attempts to map the HDAC1 and Sin3A binding sites on GRK5 were carried out using GRK5 peptide arrays. Suspected GRK5 residues involved in binding were identified and mutated, but no viable binding deficient mutants were identified. Using HDAC and Sin3A inhibitors, I show GRK5 to negatively regulate Bcl-2 transcription in a class I HDAC and, most likely, a Sin3A dependent manner. Bcl-2, an anti-apoptotic protein, is upregulated in the early stages of many colorectal cancers (CRCs) and is, at least in part, responsible for the apoptotic resistance of metastatic CRCs. Notably, GRK5 expression levels are low in the colon cancer cell line, HT-29, in comparison to other tumour-derived cancer cell lines and overexpressing the kinase in HT-29 cells by cDNA transfection increases the susceptibility of these cells to chemotherapeutically induced apoptosis. Furthermore, overexpression of an inhibitor of miR-135a, a micro RNA (miRNA) that is upregulated in CRC, increases endogenous GRK5 expression as well as apoptosis in HT-29 cells, highlighting the potential use of a miR-135a inhibitor as a novel therapeutic strategy to treat CRC patients.

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To Jonny, it's been the toughest year yet, but definitely the most rewarding. Your love, patience and encouragement have been invaluable and I am forever grateful to have you by my side.

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Abbreviations

5-FU: 5-Fluorouracil

7-AAD: 7-Aminoactinomycin D

AD: Alzheimer's disease

AdGRK5-NT: Adenoviral GRK5-N-terminal construct

ANF: Atrial natriuretic factor

AngII: Angiotensin II

Apaf1: Apoptosis protease-activating factor 1

APC: Adenomatous polyposis coil protein

ATM: Ataxia telangiectasia mutated protein

ATR: Ataxia telangiectasia and Rad3-related protein

$AT_{1A}R$: Angiotensin type 1A receptor

AUKA: Aurora A kinase

β_1AR: β_1 -adrenergic receptor

Bcl-2: B cell lymphoma protein 2

Bcl-3: B cell lymphoma protein

Bcl-xl: B cell lymphoma-extra large protein

BH: Bcl-2 homology

BSA: Bovine serum albumin

C: Carboxyl

Ca^{2+}: Calcium

CaM: Calmodulin

cAMP: cyclic AMP

CaMKII: Calmodulin kinase II

ChIP: Chromatin immunoprecipitation

CMV: Cytomegalovirus

CRC: Colorectal cancer

CTPB: Carboxyl-terminal polybasic domain

CVD: Cardiovascular disease

CMV: Cytomegalovirus

CRM1: Chromosome region maintenance 1 protein

DDB1: DNA damage-binding protein 1

DBD: DNA binding domain

DMEM: Dulbecco's modified Eagle's medium

DMSO: Dimethylsulphoxide

DOXO: Doxorubicin

DSB: Double stranded breaks

dsRNA: double stranded RNA

DTC: Differentiated thyroid carcinoma

ECL: Enhanced chemiluminescence

E. coli: Escherichia coli

EGF: Epidermal growth factor

EGFR: Epidermal growth factor receptor

ERK: Extracellular signal-regulated kinase

FACS: Fluorescence-activated cell sorting

G2/M: Growth 2/mitosis

GBM: Gliobastoma multiforme

GFP: Green fluorescent protein

GPCR: G protein-coupled receptor

Grb2: Growth factor receptor-bound protein 2

GRK: G protein-coupled receptor kinase

GRK5-NT: G protein-coupled receptor kinase 5 – amino terminal

GSC: GMB initiating cells with stem cell markers

GST: Glutathione S-transferase

GSK3β: glycogen synthase kinase-3β

GTP: Guanosine triphosphate

HAT: Histone acetyltransferase

HCM: Hypertrophic cardiomyopathy

HCR: Highly conserved region

HDAC: Histone deacetylase

HEK293: Human embryonic kidney 293

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid

HEp2: Human epithelial cell line 2

HID: HDAC interaction domain

IB: Immunoblot

IκB: Inhibitor of κB

IKK: Inhibitor of κB kinase

IP: Immunoprecipitation

IPTG: Isopropyl-β-D-thio-galactoside

Jaks: *Janus* kinases

LB: Luria broth

LPS: Lipopolysaccharide

LRP: Low density lipoprotein receptor-related protein

MAPK: Mitogen-activated protein kinase

Mdm2: Murine double minute oncogene 2

MEFs: Mouse embryonic fibroblast

MEF2: Myocyte enhancing factor 2

MEK1: MAPK/ERK kinase

miRNA: micro RNA

N: Amino

NaB: Sodium butyrate

N-CoR: Nuclear receptor co-repressor

NES: Nuclear export sequence

NFκB: Nuclear factor kappa enhancer of activated B cells

NLS: Nuclear localisation sequence

NPM1: Nucleophosmin 1

NRVM: Neonatal rat ventricular myocytes

NT: Amino-terminal region

NTPB: N-terminal polybasic domain

NTT: Normal thyroid tissue

NuRD: Nucleosome remodeling and deacetylating complex

PAH: Paired amphipathic helix

PARP-1: Poly [ADP-ribose] polymerase 1

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PD: Parkinson's disease

PDE4D5: family 4 cAMP-specific phosphodiesterase, subfamily D, isoform 5

PDGFRβ: Platelet-derived growth factor receptor

PH: Pleckstrin homology

PIP_2: Phosphatidylinositol 4,5-bisphosphate

PKA: cAMP-dependent protein kinase

PKC: Protein kinase C

PKD: Protein kinase D

PKR: Double stranded RNA (dsRNA)-dependent protein kinase

PLC: Phospholipase C

PLK1: Polo-like kinase 1

PMSF: Phenylmethanesulfonyl fluoride

RT-PCR: Reverse transcription – polymerase chain reaction

qRT-PCR: Quantitative reverse transcription – polymerase chain reaction

RACK1: Receptor for Activated C-Kinase 1

RAR: Retinoic acid receptor

Rb: Retinoblastoma protein

REST: RE1 silencing transcription factor

RGS: Regulator of G protein signalling

RH: RGS homology

SANT: Swi3, Ada2, N-CoR, and TFIIB domain

SDS: Sodium dodecyl sulphate

SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Shp2: SH2-domain-containing protein tyrosine phosphatase-2

Sin3: Swi-independent 3 protein

shRNA: Short hairpin ribonucleic acid

siRNA: Small inhibitory ribonucleic acid

SMC: Smooth muscle cell

SMRT: Silencing mediator of retinoic and thyroid hormone receptor

SNP: Single nucleotide polymorphism

SOC: Super-optimal broth with catabolite repression

Src: Sarcoma virus homologue

Stats: Signal Transducers and Activators of Transcription

T_3R: Thyroid hormone receptor

TAC: Transverse aortic constriction

TAT: Transactivator of transcription

TBL1: Transducin β-like protein 1

TBS: Tris-buffered saline

TEMED: Tetramethylethylenediamine

TIG1: Tazarotene-induced gene 1

TIEG2: Transforming growth factor β inducible early response gene 2

TLR4: Toll-like receptor 4

TNFα: Tumour necrosis factor-α

TNFR: Tumour necrosis factor receptor

TPL2: Tumour progression locus 2

TSA: Trichostatin A

TSH: Thyroid stimulating hormone

TTBS: Tween/Tris-buffered saline

Chapter 1. Introduction

1.1 G protein-coupled receptor kinase (GRK) structure

The GRK family of serine/threonine kinases were discovered in the 1990s and classified into three subfamilies based on sequence homology [1]. Figure 1.1 illustrates the GRKs grouped according to subfamily. The GRK1 subfamily includes GRK1 and GRK7, which are expressed in the retina. GRK1 is additionally expressed in the pineal gland [2]. The GRK2 and GRK4 subfamilies, comprising GRK2 and GRK3, and GRK4, 5 and 6 respectively, are ubiquitously expressed, with the exception of GRK4 that is exclusively expressed in the testes, kidney and cerebellum [3-6].

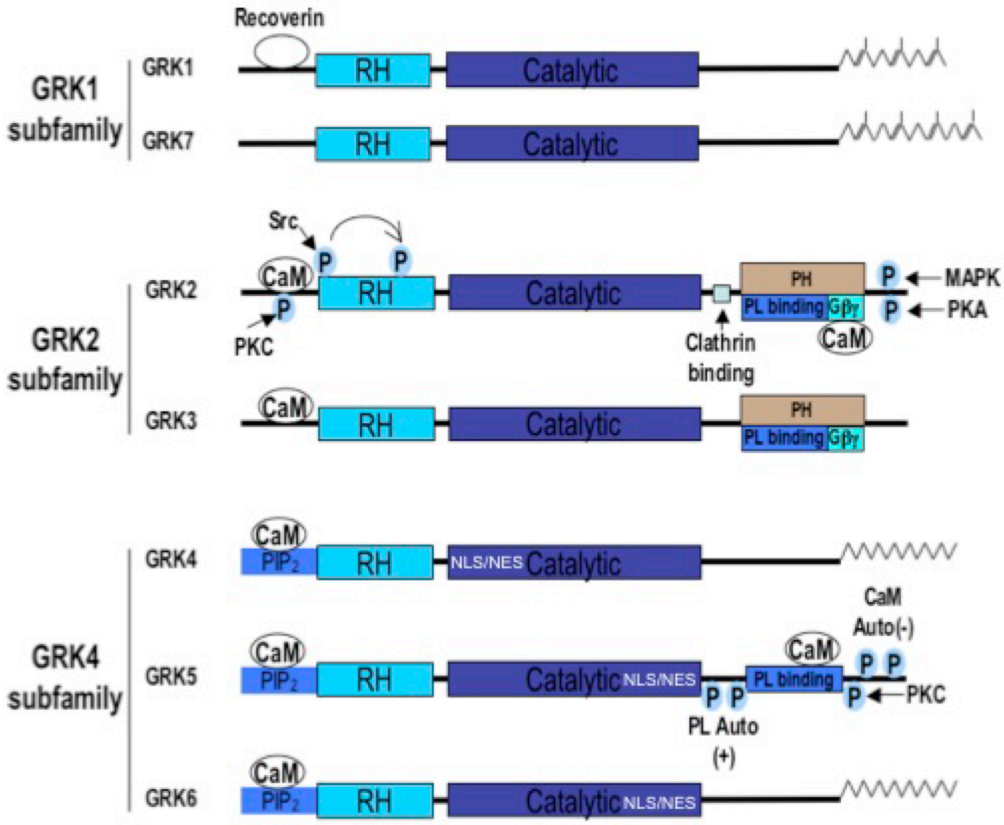


Figure 1.1 GRK subfamily organisation

The schematic shows the multi-domain structure of the GRKs along with key regulatory and interaction sites that have been mapped. The lipid modifications depicted in the carboxy-termini are farnesylation (GRK1), geranylgeranylation (GRK7) and palmitoylation (GRKs 4 and 6). RH, regulator of G protein signalling homology domain; CaM, calcium/calmodulin; NLS, nuclear localisation sequence; NES, nuclear export sequence; PL, phospholipid, PH, pleckstrin homology; auto (±), stimulatory or inhibitory autophosphorylation sites. Adapted from [7].

The GRKs have a common, multi-domain, structure with similar amino (N) and catalytic domains, as shown in Figure 1.1. An N-terminal region of approximately 183–188 residues precedes the catalytic domain and includes a region homologous to regulator of G protein signalling (RGS) proteins, known as the RGS homology (RH) domain. While RGS domains increase the inherent guanosine triphosphate (GTP) hydrolytic activity of Gα subunits of heterotrimeric G proteins, the RH domains do not posses this function [7]. The RH domains span approximately 120 residues and while they are a common N-terminal feature of the GRKs, these domains only share approximately 27% sequence identity. The RH domains of GRK2 and GRK3 bind activated members of the Gα protein family, specifically Gαq, Gα11 and Gα14 and act to sequester Gα proteins from effector proteins. Additionally, the RH domain of GRK2 binds adenomatous polyposis coli (APC) [8] and interactions of this RH domain with the kinase domain and carboxyl (C)-terminal pleckstrin homology (PH) domain may work to regulate kinase activity. The RH domains of GRK5 and GRK6 have not been reported to interact with Gα proteins and their binding partners, like the RH binding partners of the other GRKs, have not been identified [6]. In addition to the RH domain, the N-termini of the GRK4 subfamily also contain a phosphatidylinositol (4,5) biphosphate 2 (PIP_2)

binding site, which flanks the RH domain. This region serves to localise the kinase to the membrane and enhances receptor-mediated phosphorylation. The N-terminal PIP_2 binding site overlaps with a calcium/calmodulin (Ca^{2+}/CaM) binding site, such that these binding events are thought to be mutually exclusive [7].

The central serine/threonine kinase domain consists of around 320 residues and has approximately 42% sequence identity across the GRKs [9]. GRK5, as well as GRK4 and GRK6, contain a functional nuclear localisation sequence (NLS) and nuclear export sequence (NES) within their catalytic domain, as shown in Figure 1.1 [10, 11]. Mutation of the basic residues in the GRK5 NLS, Arg388–Glu395 (RKEKVKRE to AAEAVAAE) to generate a GRK5ΔNLS mutant, results in the nuclear exclusion of the kinase without affecting its catalytic activity [10, 12]. The GRK1 subfamily contain putative NLS and NESs located towards the N-terminus of the kinase domain [11, 13]. GRK5 and GRK6 share a conserved NLS located at the C-terminus of the catalytic domain, whereas the NLS of GRK4 is homologous to that of the GRK1 subfamily kinases.

The GRK C-termini show very little sequence homology, but each subfamily contains similar domains that mediate membrane binding. The GRK1 subfamily kinases contain short CAAX sequences that mediate prenylation to facilitate plasma membrane binding [14]. The GRK2 subfamily have PH domains, the C-terminus of which binds PIP_2 and the N-terminal portion binds Gβγ. Both proteins bind cooperatively to the PH domain and coordinately trigger the re-localisation of these soluble kinases to the cell membrane following agonist stimulation. Agonist occupation of G protein-coupled receptors (GPCRs) leads to the dissociation of

Gβγ from heterotrimeric G proteins, which are then free to bind the PH domains of GRK2 and GRK3. PIP_2 and Gβγ contribute synergistically to the membrane localisation of GRK2, which leads to enhanced GRK-mediated receptor phosphorylation following the allosteric activation of the GRKs by agonist occupied GPCRs [15-17]. In contrast to the GRK2 subfamily, GRK4 and GRK6 are palmitoylated, a post-translational modification that promotes membrane association [15]. GRK5 contains positively charged residues in its C-terminus to mediate phospholipid binding, which in turn stimulates autophoshorylation and activation of the kinase. GRK5 is autophosphorylated at Ser484 and Thr485. While autophosphorylation does not enhance the catalytic ability of GRK5 towards soluble peptides, it is necessary for GRK5-mediated phosphorylation of receptor substrates and is considered a mechanism of GRK5 membrane localisation [18]. GRK5 also contains a C-terminal Ca^{2+}/CaM binding site. The binding of Ca^{2+}/CaM to either the N- or C-terminal sites regulates the subcellular localisation of GRK5. This is discussed in more detail in section 1.3.3.

1.1.1 Crystal structures of the GRKs

The Tesmer group has solved the crystal structures of GRK1, GRK2 and GRK6. The GRK2 crystal structure has been solved alone, in complex with Gβγ and in complex with Gβγ and Gαq [19-23]. Similarities between the GRK crystal structures highlights the conserved nature of the RH-kinase domain interface, which may function to correctly orientate membrane binding elements of the GRKs, or stabilise the inactive conformation of the kinase. Interestingly, in all of the GRK crystal structures, the kinase domain adopts an open, inactive conformation, similar to

protein kinase A (PKA). It is suggested that GPCR activation of the GRKs may induce kinase domain closure.

GRK6 is the most ubiquitous member of the GRK4 subfamily and shares 70.1% sequence identity with GRK5 [24], which is the main kinase that I focus on in my thesis. I will therefore use the crystal structure of GRK6 to make reasonable inferences regarding the tertiary structure of GRK5. In contrast to GRK2, GRK6 crystallises as a dimer, via a surface of its RH domain that is shared among the GRK4 and GRK1 subfamily members [19]. The Tesmer group assume that GRK6 dimerisation is an artefact of crystallisation, resulting from high GRK6 protein concentrations in the crystals. The group explain that results from size exclusion chromatography and sedimentation equilibrium analysis suggests that soluble, palmitoylation-deficient GRK6 is monomeric. Furthermore, dimerisation is not required for receptor phosphorylation; three GRK6 constructs, each with a key residue involved in dimerisation mutated to alanine, were expressed in COS-1 cells and were all capable of phosphorylating rhodopsin to the same degree as wildtype GRK6 and the overexpression of double mutants only slightly impinged receptor phosphorylation [19]. Contrary to the opinion of the Tesmer group, Lan Ma's group propose GRK5 to dimerise in a cellular setting and suggest a role for the self-association of the kinase in promoting F-actin bundling at the cell membrane. In HEK293 cells, the Ma group showed Flag-GRK5 to co-immunoprecipitate with green fluorescent protein (GFP) tagged GRK5, whereas Flag-GRK2 did not. These data suggest that GRK5 may not just dimerise but could also form oligomers. The group propose that a GRK5 dimer works to crosslink F-actin into bundles via its C-terminal F-actin binding domain [25].

Based on these data, I would predict that while dimerisation may not be involved in receptor phosphorylation, GRK5 could act as a dimer to promote other cellular functions. GRK5 has a plethora of substrates and binding partners, extending the role of the kinase far beyond that of GPCR phosphorylation, the best-known function of the GRKs. As will be discussed in section 1.3.1.4, the kinase activity of GRK5 is not required for its role in F-actin bundling, implying that GRK5 dimerisation may be important for kinase-independent GRK5 functions. GRK5 contains a functional NLS that, in conjunction with an N-terminal polybasic domain (NTPB), is required for DNA binding [11]. The crystal structure of GRK6A orients the NLS and NTPB at opposing ends of the dimer structure with the NLS located at the extremities while the NTPB domains are located at the dimer interface. Dimers of GRK5 may therefore wrap around the DNA helix with the NLS and NTPB domains forming direct DNA contacts [11, 19]. So while GRK5 dimerisation may not be required for the phosphorylation of GPCRs, or even of non-receptor substrates, I propose that there may well be a functional role for the GRK5 dimer, potentially to catalyse F-actin bundling and DNA binding.

1.2 GRK function: modulating G protein signaling

GRKs were discovered for their role in negatively regulating G protein mediated signalling via GPCRs. Agonist binding to GPCRs triggers a conformational change in the receptor, such that it serves as a guanidine nucleotide exchange factor for its associated heterotrimeric G protein, catalysing the exchange of guanosine diphosphate for GTP on the G protein Gα subunit, thereby initiating G protein signalling. Following agonist binding, GRKs phosphorylate the receptor, enabling β-arrestin protein binding and the initiation of multiple intracellular signalling pathways,

one of which negatively regulates G protein signalling [15]. The role of GRKs as coordinators of GPCR signalling is perhaps under appreciated, with the main focus generally being on the β-arrestin adapter proteins, whose binding sterically inhibits G protein coupling to the receptors and triggers GPCR internalisation [26].

An analogy can be drawn between the signalling pathways initiated by GRKs downstream of GPCR activation and those initiated by *Janus* kinases (Jaks) downstream of haematopoietin cytokine receptors. [27]; Jaks provide the key link between extracellular ligand binding to cytokine receptors and intracellular protein phosphorylation, much like the GPCR/GRK relationship [28]. Jak and GRK mediated phosphorylation ensues following agonist occupation of receptors. Much like GRK5, whose phospholipid-stimulated autophosphorylation is necessary for its receptor-directed kinase activity, Jaks must first be transactivated via tyrosine phosphorylation. Following their phosphorylation and activation, GRKs and Jaks in turn phosphorylate their respective receptor targets. GRKs phosphorylate GPCRs at serine and threonine residues usually located in the third intracellular loop or the C-terminal tail, which are exposed following agonist binding [15]. Phosphorylated residues of GPCRs and cytokine receptors then act as binding sites for adapter proteins, β-arrestin and Signal transducers and activators of transcription (Stats), respectively. Docking of β-arrestins or Stat recruitment, plays a key role in negative feedback. β-Arrestin binding to GPCRs results in receptor desensitisation, as the coupling of heterotrimeric G proteins to the receptor is prevented, as is subsequent G protein-mediated signalling. Furthermore, the β-arrestin-mediated recruitment of proteins including the clathrin adapter, AP2, triggers receptor internalisation [29].

My thesis focuses on GRK5 and its role in the nucleus as opposed to at the plasma membrane. I will, therefore, proceed to discuss additional functions of GRK5 distinct from GPCR phosphorylation, but I will compare and contrast with other GRK family members where relevant.

1.3 Functions of GRK5

In recent years, the role of GRKs has extended beyond the initiation of GPCR desensitisation. GRK5 is now widely recognised as an independent signalling coordinator, regulating a multitude of signalling pathways at the membrane, as well as in the nucleus. As more GRK-specific substrates and binding partners are discovered, distinct and specific roles for the kinases are being identified. Figure 1.2 depicts the multiple substrates and binding partners of GRK5 identified to date, detailing some of the physiological and pathophysiological processes that are regulated by the kinase.

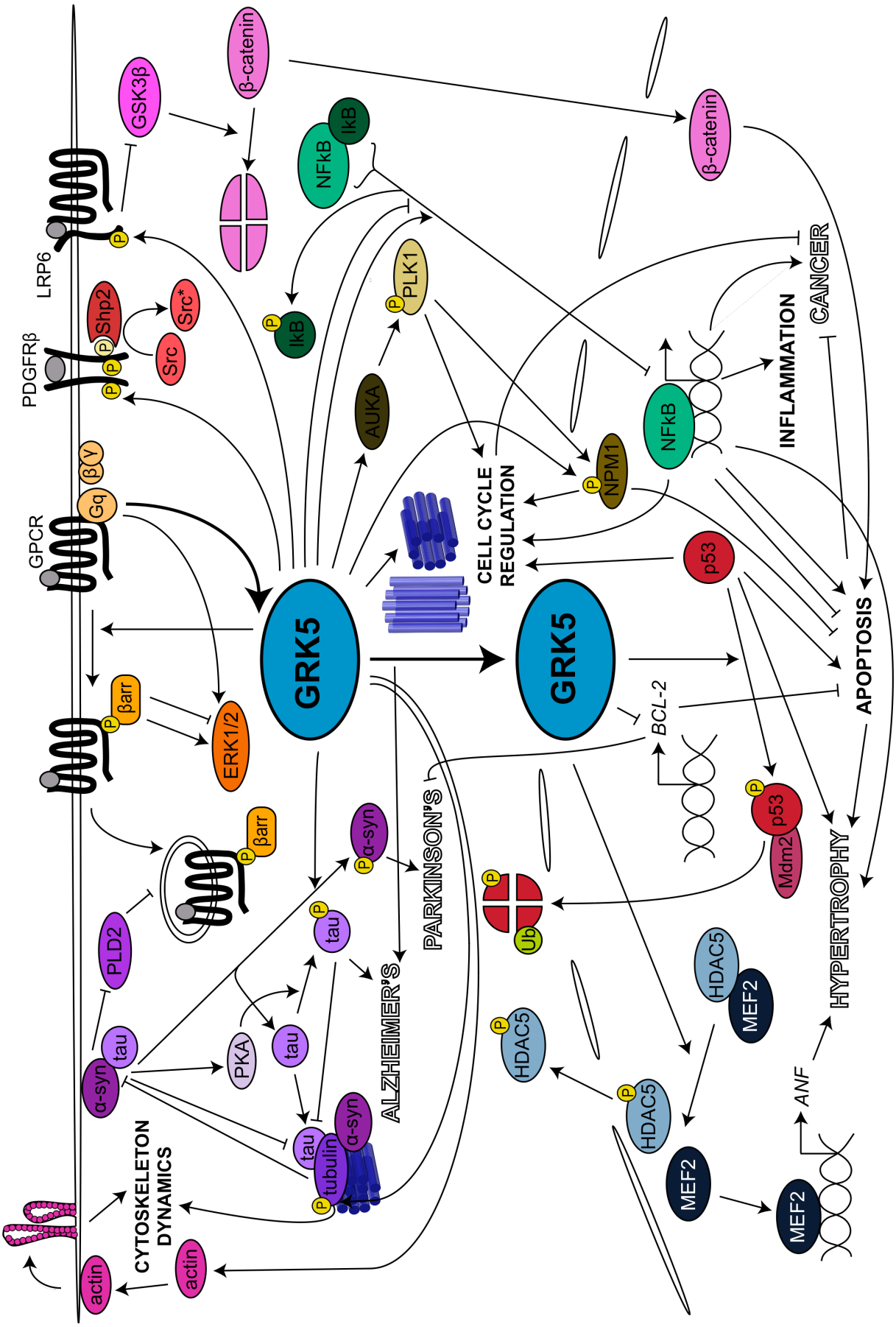

GRK5
GRK5
GPCR
Gq
βγ
βarr
PDGFRβ
LRP6
Shp2
Src
Src*
GSK3β
β-catenin
β-catenin
NFkB
IkB
IkB
PLK1
AUKA
NPM1
CELL CYCLE REGULATION
NFkB
INFLAMMATION
CANCER
p53
APOPTOSIS
BCL-2
p53
Mdm2
ERK1/2
βarr
PLD2
α-syn
tau
PKA
tau
α-syn
tau
tubulin
α-syn
ALZHEIMER'S
PARKINSON'S
Ub
HDAC5
MEF2
HDAC5
HDAC5
MEF2
MEF2
ANF
HYPERTROPHY
actin
actin
CYTOSKELETON DYNAMICS

Figure 1.2 Substrates and binding partners of GRK5

GRK5 has a range of substrates and binding partners in addition to GPCRs, which are located not only at the cell surface but also in the cytoplasm and nucleus. Starting with membrane substrates and binding partners and working from left to right: GRK5 promotes F-actin bundling at the cell membrane, leading to the formation of filopodia and dendritic branches in neurons, thus implicating GRK5 in the control of cytoskeletal dynamics. GRK5 phosphorylates α-synuclein and tubulin, promoting the dissociation of α-synuclein from tau and the formation of microtubules respectively. Serine and threonine phosphorylation is shown in bright yellow. α-Synuclein accumulates in Lewy bodies during Parkinson's disease. Accumulation of phosphorylated tau is common to Alzheimer's disease and relocalisation of GRK5 from the cell membrane is also thought to contribute to disease pathology. The best-known substrate of GRK5 are GPCRs, whose phosphorylation stimulates β-arrestin protein binding that in turn triggers receptor endocytosis and desensitisation by preventing G protein coupling to receptors. Two distinct mechanisms of ERK regulation operate downstream of activated GPCRs, either via β-arrestin or Gq. Gq-activation may also trigger the GRK5 cellular relocalisation, potentially to the nucleus (Bold arrows). GRK5 phosphorylates platelet derived growth factor receptor β (PDGFRβ), which promotes tyrosine phosphorylation (shown as pale yellow phosphate groups), to which SH2-domain-containing protein tyrosine phosphatase-2 (Shp2) binds to activate Sarcoma virus homologue (Src). The Wnt signalling receptor, low density lipoprotein receptor-related protein (LRP) 6, is phosphorylated by GRK5, which inhibits the action of glycogen synthase kinase-3β (GSK3β), thus resulting in the accumulation of β-catenin and the upregulation of Wnt target genes. Cytoplasmic GRK5 substrates and binding partners include inhibitor of kappa Bα (IκBα), whose phosphorylation activates nuclear factor kappa enhancer of activated B cells (NFκB). GRK5 is also known to inhibit NFκB signalling by preventing the phosphorylation of IκB isoform, p105. GRK5 is implicated in cell cycle control by localising at centrosomes and by directly and indirectly promoting the phosphorylation of nucleophosmin 1 (NPM1). Nuclear GRK5 substrates include histone deacetylase 5 (HDAC5), whose phosphorylation leads to the upregulation of pro-hypertrophic genes, such as atrial natriuretic factor (ANF), and p53, whose phosphorylation and degradation inhibits apoptosis and whose

activation promotes hypertrophy. GRK5 also negatively regulates B-cell lymphoma 2 (Bcl-2) gene transcription, which may be a functioning mechanism in Parkinson's disease.

1.3.1 Functions of GRK5 at the plasma membrane

1.3.1.1 Regulation of extracellular signal-regulated kinase (ERK) signalling via β-arrestins

The β-arrestin proteins are appreciated as independent signalling mediators, playing a signalling role distinct from GPCR desensitisation and internalisation. The canonical means of β-arrestin activation is reliant upon GRK-mediated phosphorylation of agonist occupied receptors. Moreover, the signalling pathway mediated by β-arrestin proteins can be further determined by the specific GRK that catalyses GPCR phosphorylation.

ERK is a downstream effector kinase of the angiotensin type 1A receptor ($AT_{1A}R$), activated in response to receptor stimulation. ERK1/2 activation occurs by two distinct and parallel pathways, via Gq or β-arrestin 2, which GRK2 and GRK3, and GRK5 and GRK6 regulate respectively. Gq-mediated signalling requires protein kinase C (PKC)-mediated ERK1/2 activation. Stimulation of the $AT_{1A}R$ with angiotensin II (AngII) in HEK293 cells, however, results in significant ERK1/2 activation that is only approximately 60% diminished by treatment with the PKC inhibitor, Ro-31-8425, thus implying an additional G protein-independent means of regulating this pathway. Indeed, the remaining ERK1/2 activation was abolished following knockdown of β-arrestin 2 or GRK5 [30]. β-Arrestin 2 acts as a scaffold for the mitogen activated protein kinase (MAPK) cascade, enabling the phosphorylation and activation of ERK1/2 by directly binding to the MAPK, Raf1, as

well as to ERK1/2, which mediates an indirect interaction of MAPK/ERK kinase 1 (MEK1) with β-arrestin 2 [31]. Phosphorylation of the $AT_{1A}R$ by either GRK2 or 3, or GRK5 or 6, stimulates a different arm of the signalling pathway. Phosphorylation by GRK2 or 3 promotes receptor internalisation and thereby reduces ERK1/2 activation by preventing G protein coupling, whereas phosphorylation by GRK5 or 6 stimulates β-arrestin 2-mediated ERK1/2 activation. Knockdown of GRK2 or GRK3, or the overexpression of GRK5 or GRK6 enhanced β-arrestin 2-mediated ERK1/2 activation in HEK293 cells [32]. The contrasting effects mediated by GRK2/3 and GRK5/6 therefore suggest that the GRK2 and GRK4 subfamily kinases recognise specific receptor conformations and phosphorylate different serine and threonine residues of the $AT_{1A}R$. GRK5 subfamily-dependent stabilisation of specific receptor conformations is presumably reflected in different conformations of receptor bound β-arrestin 2 and thus differential recruitment of binding partners. This suggests a function for GRK5 that extends beyond the canonical role of GRKs in controlling receptor internalisation and suggests that GRK5 can in fact function as a positive regulator of ERK1/2 signalling. This function for GRK5 is not specific to the $AT_{1A}R$ but is also operative at the β-adrenergic receptor and vasopressin V2 receptor [33, 34].

As discussed above, the parallel signalling pathways evoked from the $AT_{1A}R$ suggests discrete active conformations of the receptor. Biased agonists, which selectively activate specific signalling pathways downstream of receptors, pave the way for therapeutic advances. β-Arrestin-dependent signalling downstream of the β_1 adrenergic receptor (β_1AR) is cardioprotective, whereas classical G protein signalling is injurious, with the different cardiovascular phenotypes attributed to the

activation of distinct ERK signalling events [35]. G protein-mediated signalling promotes rapid and transient ERK activation leading to its nuclear relocalisation, which activates transcription of the early growth response 1 gene. In contrast, β-arrestin-mediated signalling results in slower activation of ERK [35]. ERK signalling is initiated following the transactivation of epidermal growth factor receptors (EGFRs), which occurs following the GRK5/6-mediated phosphorylation of β_1AR and results in cardioprotection. Transgenic mice overexpressing a mutant receptor lacking the GRK phosphorylation sites develop left ventricular dilation and myocyte apoptosis following chronic stimulation with the β-adrenergic receptor agonist, isoproterenol. Additionally, wildtype mice stressed with isoproterenol and treated with the EGFR inhibitor, erlotinib, have impaired cardiac function [36]. Chronic stimulation of β-adrenergic receptors and AT_{1A}Rs are involved in heart disease pathogenesis, such that most treatments function to block signalling from these receptors, inhibiting both G protein and β-arrestin signalling pathways. The development of biased ligands that preferentially inhibit the G protein arm and/or stimulate the β-arrestin signalling arm may have a more beneficial effect. The synthetic AT_{1A}R ligand, [Sar1, Ile4, Ile8]-Ang and the β-blocker, carvedilol, both work to this effect, with cardioprotective effects demonstrated *in vivo* and in human patients respectively [35].

Interestingly, and in contrast to the previously described example, GRK5 negatively regulates Sarcoma virus homologue (Src)/ERK signalling downstream of the serotonin receptor, 5-HT_4, in both HEK293 cells and in neurons. Overexpression of GRK5 and the 5-HT_4 receptor in HEK293 cells attenuates ERK and Src phosphorylation. Overexpression of a transactivator of transcription (TAT) cell-

permeable peptide fused to the GRK5 binding site on the receptor, which therefore prevents the GRK5/5-HT_4 interaction, increased ERK activation in both HEK293 cells and in neurons. It was subsequently demonstrated that the GRK5-mediated inhibition of the ERK signalling pathway downstream of 5-HT_4 activation required GRK5-mediated phosphorylation of both the receptor and β-arrestin 1 [37]. GRK5 thereby differentially regulates ERK signalling, possibly in a manner that is dependent on the upstream receptor as well as the β-arrestin isoform recruited to the phosphorylated, activated receptor.

1.3.1.2 Platelet derived growth factor receptor-beta (PDGFRβ) regulation

GRK2 is the dominant GRK phosphorylating the tyrosine kinase receptor, PDGFRβ following acute stimulation in endothelial cells. Prolonged receptor activation in mouse aortic smooth muscle cells (SMCs), however, results in down-regulation of GRK5, while GRK2 expression levels are unaffected [38]. The two kinases regulate PDGFRβ function via distinct mechanisms, phosphorylating different receptor serine residues. GRK5-mediated seryl phosphorylation results in subsequent phosphorylation of PDGFRβ at Tyr1009, to which the phosphatase SH2-domain-containing protein tyrosine phosphatase-2 (Shp2) binds (Figure 1.2). Autophosphorylation of PDGFRβ at Tyr1021 is reduced, which is the binding site for phospholipase C (PLC)-γ1, such that this signalling pathway is inhibited. GRK5 thereby desensitises inositol phosphate signalling via PLC-γ1, but promotes Src signalling, as Shp2 dephosphorylates Src at its autoinhibitory residue, Tyr527. Overexpression of GRK5 in SMCs thus leads to greater Src activation but interestingly not ERK1/2. In a pathological setting where PDGFRβ is upregulated, so are GRK5 expression levels; atherosclerotic arteries of mice contain

substantially more GRK5 and PDGFRβ per unit of α-SMC actin compared to wildtype mice, as assessed by immunofluorescence. Like PDGFRβ, GRK5 may therefore also have a role in atherosclerosis and based on these data I would predict the kinase to promote disease pathogenesis [38].

1.3.1.3 Regulation of Wnt signalling

Wnt ligands bind 7-transmembrane, Frizzled receptors, which heterodimerise with the single transmembrane, low density lipoprotein receptor-related proteins (LRP) 5 and 6 to activate the canonical Wnt signalling pathway that regulates organogenesis and tissue regeneration [39]. Activation of Wnt signalling receptors leads to the inhibition of the cytosolic protein, glycogen synthase kinase-3β (GSK3β), which is part of a destruction complex that includes casein kinase I, the scaffolding protein, axin, and the tumor suppressor gene product, APC, and functions to degrade β-catenin. Wnt signalling, therefore, subsequently stabilises β-catenin, such that it translocates to the nucleus and acts as a transcriptional co-activator, leading to the upregulation of target genes including c-Myc and cyclin D1, which promote cell proliferation [40]. GRK5 and GRK6 stimulate Wnt signalling by phosphorylating serine and threonine residues within the five PPPSP motifs in LRP6 (Figure 1.2). Knockdown of GRK5 in zebrafish causes a simultaneous reduction in Wnt signalling, noted by a reduction in β-catenin protein levels, as well as reduced mRNA levels of Wnt target genes [41].

A role for β-arrestins in the same signalling pathway has also been identified, regulating Wnt signalling in an opposing manner to GRK5 and GRK6; β-arrestin 2 promotes Wnt5A-stimulated internalisation of the Frizzled 4 receptor [42]. The

opposing roles played by GRK5 and β-arrestin 2 in regulating Wnt signalling support the notion that GRK5 can function as an independent signalling mediator, in a role distinct from its regulation of β-arrestin mediated signalling. Furthermore, the role of GRK5 and GRK6 in phosphorylating LRP6 to activate Wnt signalling solves a previously unexplained paradox. GSK3β was proposed to translocate to the membrane and bind LRP6 via axin, to catalyse receptor phosphorylation at the PPPSP motif. Axin binding, however, is dependent on the phosphorylation of the motif, which suggests that receptor phosphorylation and axin recruitment must be GSK3β independent. The involvement of GRK5 and GRK6 in the Wnt signalling pathway suggests these kinases function upstream of GSK3β to activate the receptor. The implications of these findings by the Lefkowitz and Chen groups may potentially be vast, as Wnt target genes are involved in major developmental processes and deregulated Wnt signalling has been implicated in numerous cancers, including colorectal, which will be discussed in section 1.4.3.

1.3.1.4 Cytoskeletal regulation

GRK5 is associated with cytoskeletal regulation via interactions with and regulation of α-synuclein, tubulin and actin. The function of α-synuclein is not entirely clear but it is known to bind to the cell membrane via phospholipids and play a role in vesicular trafficking. The Benovic group showed GRK5-mediated phosphorylation of α-synuclein at Ser129 reduces phospholipid binding, which is thought to be responsible for a loss in phospholipase D2 inhibition [43]. Phospholipase D2 promotes vesicle formation indirectly, firstly by generating phosphatidic acid from phosphatidylcholine hydrolysis, which stimulates vesicle assembly and secondly via its role in the rearrangement of the cortical actin cytoskeleton. GRK5-mediated

α-synuclein phosphorylation may therefore enhance receptor endocytosis. In addition to its potential role coordinating vesicular trafficking, α-synuclein also has a role in cytoskeletal regulation in neurons, via interactions with the microtubule-associated protein, tau. α-Synuclein binds soluble tau at its microtubule binding domain and this interaction is blocked by tubulin. Furthermore, α-synuclein stimulates PKA-catalysed phosphorylation of tau at sites including Ser262 and Ser356, which also inhibits the association of tau with microtubules (Figure 1.2) [44]. The GRK5 phosphorylation site on α-synuclein overlaps with the tau binding site, such that GRK5-mediated phosphorylation of α-synuclein could alter the dynamics of microtubule assembly by disrupting its interaction with tau, potentially resulting in a higher proportion of microtubule associated tau. Moreover, α-synuclein and GRK5 both interact with tubulin, which polymerises to form microtubules and GRK5-mediated tubulin phosphorylation may promote microtubule assembly [45, 46].

GRK5 and GRK2 bind and phosphorylate soluble tubulin heterodimers as well as insoluble microtubules and immunofluorescence experiments in COS-1 cells showed co-localisation of the kinases with microtubules [46]. GRK5-mediated tubulin phosphorylation may play a role in regulating microtubule function, as phosphorylation of microtubules enhances their interaction with plasma membranes as well as with actin filaments. It is also possible that interactions with tubulin may regulate the cellular localisation of GRK5. While neither of these possibilities has been investigated, *in vitro* functional analyses performed by the Benovic group suggest that tubulin phosphorylation by GRK5 may promote microtubule assembly, as following incubation with GRKs and [γ-^{32}P] ATP,

approximately 90% of phosphorylated and thus radiolabelled tubulin was associated with insoluble microtubule pellets. Overall, therefore, GRK5 may positively regulate microtubule growth via α-synuclein and tubulin phosphorylation.

The other main component of the cytoskeleton is actin, which Lan Ma's group identified as a binding partner of GRK5. A kinase-independent role for GRK5 in promoting the membrane localisation and bundling of F-actin has been identified, as actin negatively regulates the catalytic activity of GRK5. GRK5 acts as a scaffold for F-actin bundling, coordinating its association with the membrane via PIP_2 (Figure 1.2). The C-terminal of GRK5 is responsible for actin binding while its N-terminal PIP_2 binding site targets the filaments to the plasma membrane. GRK5 thereby promotes neuronal morphogenesis by stimulating actin bundling at the membrane to form filopodia, which are essential for neurite initiation and dendrite branching and are precursors for presynaptic termini and postsynaptic spines [47]. Moreover, spine morphology is important for synaptic plasticity, which in turn controls long-term memory formation. GRK5 knockout mice, which suffer reductions in the number of hippocampal neurites and dendritic spines also have impaired long-term memory [25].

1.3.2 Functions of GRK5 in the cytosol

1.3.2.1 Cell cycle control

The Benovic group discovered GRK5 localises to centrosomes, preferentially the mother centriole, inferred by immunofluorescence experiments showing co-localisation of GRK5 with γ-tubulin, which polymerises to form centrosome

microtubules. GRK5 promotes *de novo* microtubule formation, a process known as microtubule nucleation. HeLa cells were treated with nocodozole to depolymerise microtubules and re-growth was assessed by monitoring the size of asters, which hold centrioles at the poles of the cell during mitosis. In comparison to control cells, stable GRK5 knockdown in HeLa cells significantly impaired aster size. This work directly implicates GRK5 in cell cycle regulation and indeed, GRK5 knockdown in HeLa cells results in growth 2 /mitosis (G2/M) cell cycle arrest, as assessed by flow cytometry [48]. A possible mechanism whereby GRK5 regulates the cell cycle was suggested to involve the activation of aurora A kinase (AUKA), which phosphorylates polo-like kinase 1 (PLK1), which controls the progression of cells from G2 to mitosis (Figure 1.2). In HeLa cells expressing a GRK5 short hairpin RNA (shRNA), phosphorylation of PLK1 was delayed and expression levels of phosphorylated AUKA were reduced by 50% compared to control shRNA expressing cells [48]. The G2/M arrest induced by GRK5 knockdown in HeLa cells is not only attributed to the abrogation of AUKA activity, but also to the upregulation of p53, which, as will be discussed in section 1.3.3.2, is negatively regulated by GRK5 [48, 49].

GRK5 may also be promoting cell cycle progression via its role as a nucleophosmin 1 (NPM1) kinase [50]. NPM1 is involved in cell cycle control, centrosomal duplication, mitosis as well as apoptosis and its phosphorylation by PLK1 at Ser4 is important for proper mitotic spindle assembly [50, 51]. While inhibition of PLK1 ultimately results in apoptosis, it also causes elongated and fragmented nuclei, as does the overexpression of an NPM1 S4A mutant in HeLa cells, suggesting the PLK1-mediated phosphorylation of NPM1 at Ser4 is responsible for spindle

formation, defects of which result in improper cell division. GRK5 phosphorylates NPM1 at the same site as PLK1 as well as at Thr199, albeit to a lesser extent, and combined Ser4 and Thr199 phosphorylation of NPM1 regulates centrosome duplication. The Benovic group did not investigate the implications of GRK5-mediated NPM1 phosphorylation on cell cycle control but instead focussed on the apoptotic phenotype. Their investigation into the functional consequence of GRK5 activity towards NPM1 highlighted a role for GRK5 in compensating for loss of PLK1 activity. GRK5 knockdown in HeLa cells only enhanced apoptosis following the combined inhibition of PLK1 by the inhibitor, BI2536, and knockdown of GRK5 only reduced Ser4 phosphorylation when combined with BI2536 treatment [50].

This latter study by the Benovic group does not necessarily support a role for GRK5 in regulating the cell cycle, as the effect of GRK5 knockdown on spindle formation was not studied and GRK5 does not regulate centrosomal duplication despite phosphorylating both Ser4 and Thr199 on NPM1 [48, 50]. Instead, identifying NPM1 as a substrate and binding partner of GRK5 highlights a role for GRK5 in regulating apoptosis. Indeed, GRK5 phosphorylates p53, a key activator of apoptosis, which triggers its degradation [49]. Furthermore, NPM1 is a nuclear protein, therefore implying that the functional role of GRK5 extends beyond the plasma membrane and cytoplasm. The nuclear functions of GRK5 and the role of GRK5 in regulating apoptosis are discussed in section 1.3.3.

1.3.3 Functions of GRK5 in the nucleus

In addition to its established role at the plasma membrane, as well as its role regulating the cytoplasmic protein, tubulin, GRK5 can also translocate to the

nucleus. The nuclear localisation of GRK5 was first observed in spontaneously hypertensive heart failure rat myocytes by immunofluorescence [52] and contrasts with GRK2, which is excluded from the nucleus [53]. GRK5, as well as GRK4 and GRK6, contain functional NLSs and NESs when overexpressed and are present in the nucleus of Human epithelial cell line 2 (HEp2) cells under basal conditions [10]. Similarities in amino acid sequence of the NLS and its location within the kinase domain of GRK5 and GRK6 compared to GRK4, suggests potential specificities of GRK4 subfamily nuclear functions. Furthermore, the NLS of GRK5 and GRK6 bind DNA *in vitro* in contrast to GRK4, which does not [10]. Numerous nuclear substrates and binding partners of GRK5 have been identified, enhancing the appreciation of GRK5 as a key regulator of a variety of nuclear signalling pathways, which will be discussed in this section.

The regulation of GRK5's nuclear localisation is somewhat debated, particularly the mechanism of GRK5 nuclear import and export. CaM binding to GRK5 inhibits GRK5-mediated phosphorylation of GPCRs, by disrupting binding to membrane phospholipids and has also been proposed to trigger the nuclear localisation of the kinase [54, 55]. GRK5 contains two CaM binding sites located at either ends of the kinase, between residues 20–39, as well as between residues 540–578 [7]. The Koch group propose N-terminal CaM binding to be important for regulating GRK5 nuclear import. Mutation of Trp30, located in the N-terminal GRK5 CaM binding site, to alanine (W30A) severely disrupts the nuclear localisation of the kinase in neonatal rat ventricular myocytes (NRVM) following stimulation by Gq or phenylephrine, causing the W30A mutant to adopt a very membranous localisation [56]. The Benovic group originally attributed N-terminal CaM binding to the

inhibition of GPCR phosphorylation by GRK5 [55], but a year later they showed that CaM binding to the C-terminal site is most important for inhibition of rhodopsin phosphorylation. When expressed in COS-1 cells, a GRK5 construct containing the W30A mutation phosphorylated rhodopsin to the same degree as wildtype GRK5 and furthermore, CaM still inhibited the GRK5 W30A mutant-mediated phosphorylation of rhodopsin to the same efficacy as the wildtype kinase [57]. Based on these data I would predict that CaM should still be able to disrupt membrane binding of the W30A mutant, and, if CaM binding was responsible for sending GRK5 to the nucleus, this in turn should trigger relocalisation of the kinase. This, however, does not occur, as when the Koch group overexpressed the W30A mutant in NRVM, an immunofluorescence experiment showed the mutant to be membrane bound [56]. These conflicting results therefore suggest that CaM binding may not be responsible for stimulating the nuclear import of GRK5.

In comparison to the Koch group's immunofluorescence data, a GRK5 construct containing mutated polybasic residues in the N-terminal CaM binding site (GRK5ΔNTPB) has a nuclear localisation in HEp2 cells [58]. This GRK5 construct is not only unable to interact with CaM, but interactions with PIP_2, whose binding site overlaps with the N-terminal CaM binding site, are also inhibited. Loss of PIP_2 binding may therefore potentially be responsible for triggering the relocalisation of GRK5 to the nucleus. This hypothesis fits with Koch's data that shows GRK5's nuclear localisation lies downstream of Gq. Following Gq activation, levels of PIP_2 fall due to the initiation of G protein signalling and the subsequent activation of PLC, which hydrolyses PIP_2.

A similar mechanism of disrupted membrane association causing nuclear relocalisation has been proposed for GRK6A. The nuclear localisation of GRK6A is triggered by disrupting membrane binding through the use of the palmitoylation inhibitor, 2-bromopalmitate [59]. GRK6A is localised to the plasma membrane via an amphipathic helix as well as via the palmitoylation of cysteine residues located within the last 30 amino acids of the kinase [59, 60]. A C-terminal GRK6A deletion mutant is no longer plasma membrane localised but instead is redistribution to the cytoplasm and also to the nucleus [60].

With loss of PIP_2 binding regulating nuclear import, I propose CaM binding to regulate the nuclear export of GRK5 in coordination with the NES. Gq activation or treatment of HEp2 cells with the calcium ionophore, A23187, both of which increase intracellular Ca^{2+} concentrations, stimulates nuclear export of GRK5 [10]. While the overexpression of GRK5ΔNTPB in HEp2 cells was resistant to ionophore treatment, by comparison, mutating similar residues in the C-terminal polybasic domain (CTPB) of GRK5 to perturb C-terminal CaM binding did not affect A23187-dependent nuclear export [11]. I thereby propose that binding of CaM to the N-terminus of GRK5 therefore mediates Ca^{2+}-dependent nuclear export of the kinase. N-terminal CaM binding to nuclear GRK5 is postulated to dissociate the NTPB as well as the NLS from DNA, triggering a conformational change to expose the NES, thereby triggering relocalisation of the kinase. In contrast, GRK6 is not exported following ionophore treatment, suggesting a differential means of nuclear export [11].

An obvious paradox with this proposed mechanism of nuclear export is that the Koch group observe Gq activation to stimulate nuclear localisation of GRK5 in NRVM whereas in HEp2 cells Gq activation causes nuclear exclusion of the kinase. The difference in observed subcellular localisation may be accounted for by differences in the levels of free CaM. In myocytes, most of the CaM is sequestered due to the number of calcium-binding proteins present, such that the concentration of free CaM is thus very low. According to the literature, concentrations of intracellular CaM range between 2–25 μM depending on the cell type. In myocytes 50–75 nM is free, which is only approximately 1% of total cellular CaM [61]. By contrast, in HEK cells, 50% CaM is estimated to be free [61, 62]. In myocytes, I hypothesise that when PIP_2 levels fall, GRK5 enters the nucleus and as levels of activated CaM are too low to promote its nuclear export, the kinase is observed to be nuclear by immunofluorescence. In HEp2 cells, however, activation of Gq-coupled receptors sends GRK5 to the nucleus but it is rapidly exported due to relatively high levels of activated CaM, so following receptor activation GRK5 is seen by immunofluorescence to be excluded from the nucleus. Clearly more research is required to elucidate the regulatory mechanisms controlling the subcellular localisation of GRK5.

Despite all GRK4 subfamily members containing a functional NLS, differences in their nuclear capabilities and subcellular regulation imply nuclear specific functions of the kinases. While nuclear functions of GRK6 and GRK4 are currently unreported, several GRK5 nuclear substrates and binding partners have been identified.

1.3.3.1 Nuclear factor kappa enhancer of activated B cells (NFκB) signaling

The NFκB family of transcription factors function as multifaceted regulators of cell survival mechanisms, controlling the expression of genes that affect inflammation and immunity as well as proliferation, apoptosis and tissue remodelling. There are five members of the mammalian NFκB family of proteins, which form homo and heterodimers: p50 (NFκB1), p52 (NFκB2), p65 (RelA), RelB and c-Rel. Regulation of NFκB is mostly focused on the inhibitor of κB (IκB) proteins, which serve to bind and sequester NFκB dimers in the cytoplasm. IκB proteins include IκBα, IκBβ, IκBε, IκBζ, B-cell lymphoma 3 (Bcl-3) and p105 and p100, which are the precursors of p50 and p52 respectively. Phosphorylation of IκB by the IκB kinase (IKK) at conserved “destruction box” serine residues, results in IκB ubiquitination and degradation and releases NFκB for translocation to the nucleus and subsequent upregulation of target genes [63].

Figure 1.3 illustrates the two different NFκB pathways and the roles played by GRK5 in the canonical signalling pathway. Activation of the canonical NFκB pathway (Figure 1.3A) ensues following stimulation of, among others, tumour necrosis factor receptors (TNFRs), toll-like receptor 4 (TLR4) and the interleukin receptor-1. Instigation of signalling cascades culminates in the activation of IKKβ (IKK2), which resides in complex with IKKα (IKK1) and the regulator, IKKγ (NEMO). Substrates of IKKβ include IκBα. The identification of NLS and NES domains in the IκBα/p65/p50 complex suggests its continual nuclear-cytoplasmic shuttling. The complex, however, remains cytoplasmic in the steady state due to the combined exposure of the NES on IκBα, as well as masking of the NLS on p65 [64]. In contrast, the non-canonical signalling pathway (Figure 1.3B) involves activation of

IKKα, is independent of NEMO and results in the proteolysis of p100, which leaves p52/RelB heterodimers free to shuttle to the nucleus. Phosphorylation of IKKα is catalysed by NFκB inducing kinase. IKKα and IKKβ can also mediate crosstalk with other signalling pathways including MAPKs, which are activated by GPCRs as well as toll-like receptors.

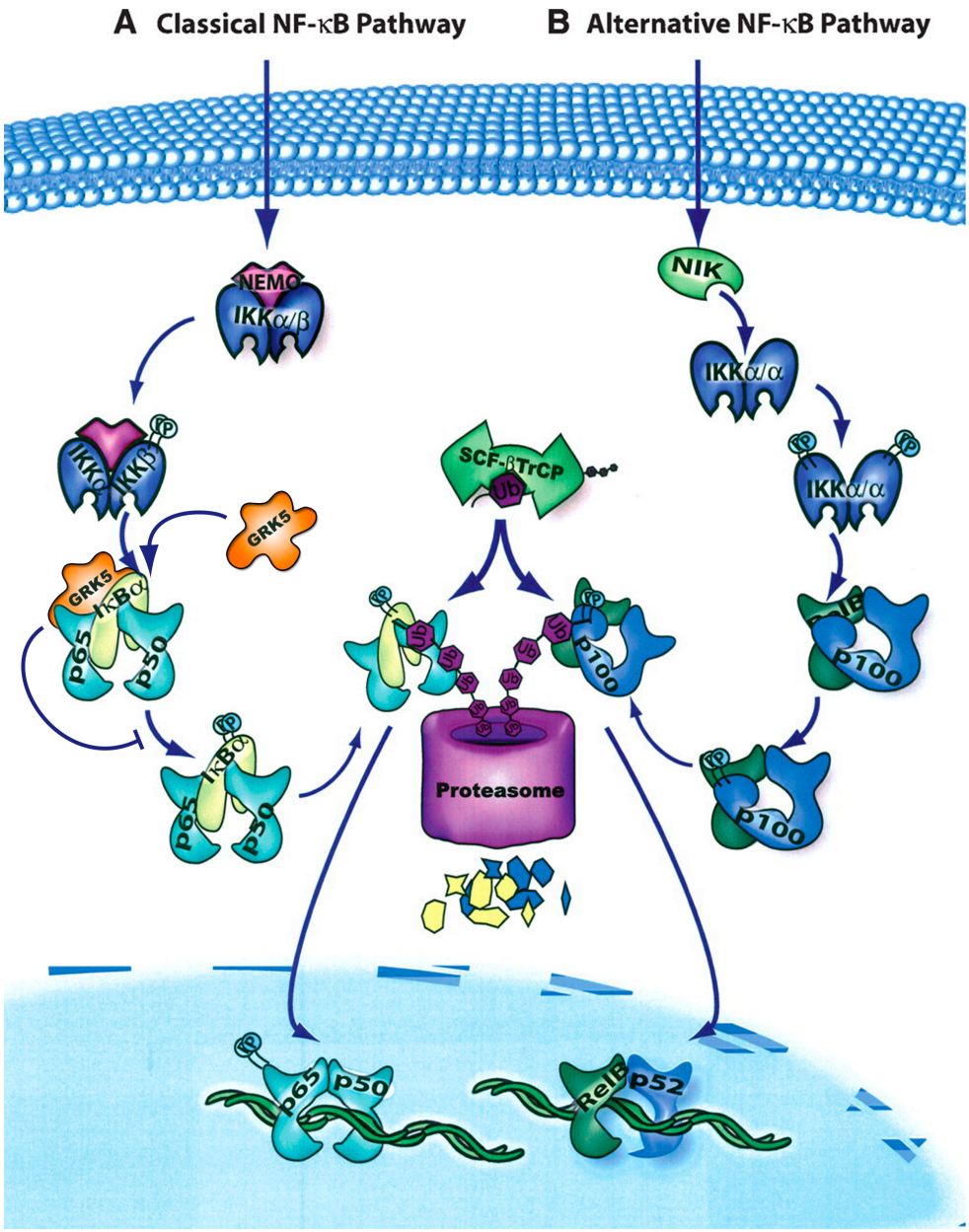


Figure 1.3 NFκB signalling network

A. The classical pathway is mediated by IKKβ and results in phosphorylation of IκBα. GRK5 can phosphorylate IκBα following the activation of TNFRs but it binds and inhibits IκBα phosphorylation following TLR4 stimulation. **B.** The non-canonical pathway begins with IKKα phosphorylation by NFκB inducing kinase (NIK) and results in degradation of the IκB protein, p100. p52/RelB heterodimers then translocate to the nucleus. Adapted from [64].

The link between GRKs and NFκB regulation stemmed from the identification of arrestin proteins as indirect NFκB signalling mediators. The elucidation of novel functions of the GRKs distinct from receptor phosphorylation sparked the expansion of these studies to identify whether the GRKs could also feature in the NFκB pathway and indeed, GRK5 is an important regulator of NFκB activity. Mechanistically, this is due to interactions of GRK5 with IκB and in some cases GRK5-mediated IκB phosphorylation, the functional consequences of which may depend on the cell type, the initiating stimulus and the IκB isoform involved (Figure 1.2).

Work by the Iaccarino group has shown GRK5 to bind directly to IκBα in endothelial cells, triggering translocation of the complex to the nucleus, which thereby reduces NFκB-mediated transcription. Interestingly, GRK5 binds to the N-terminus of IκBα, which may mask the NES located in its C-terminus, thus sequestering IκBα/NFκB in the nucleus. The GRK5/IκBα relocalisation can be triggered by isoproterenol, which activates β-adrenergic receptors, suggesting that GRK5 regulates IκBα following GPCR activation. The GRK5/IκBα interaction alone is responsible for inhibiting NFκB, rather than GRK5 kinase activity; overexpression of a kinase dead GRK5 mutant, GRK5ΔK215R, in bovine aortic endothelial cells, behaved like GRK5

wildtype with regards to IκBα nuclear accumulation and the phosphorylation state of IκBα remained constant following wildtype GRK5 overexpression. The binding site on IκBα has been mapped to the RH domain in the N-terminus of GRK5 and the overexpression of this domain alone (GRK5-RH) resulted in reduced NFκB stimulation following lipopolysaccharide (LPS) stimulation of TLR4. GRK2 can also co-immunoprecipitate with IκBα but fails to regulate NFκB-mediated transcription [65].

Combined studies by the Benovic and Parameswaran groups have identified contrasting roles for GRK5 in regulating NFκB signalling pathways. In keeping with findings by the Iaccarino group, Benovic and Parameswaran showed GRK5 to negatively regulate TLR4-induced ERK activation by inhibiting the phosphorylation of the NFκB precursor, p105, in macrophages [66]. The MAPK kinase kinase protein, tumour progression locus 2 (TPL2) is sequestered by p105 in an inactive state; following phosphorylation and subsequent degradation of p105, TPL2 is free to initiate the MAPK signalling cascade that culminates in ERK1/2 activation [67]. GRK5 binds directly to the C-terminus of p105 at IκBγ, blocking its phosphorylation by IKK. GRK2 is also capable of p105 binding but specific to GRK5 is its ability to regulate p105 phosphorylation; knockdown of GRK5 as opposed to GRK2 increases p105 phosphorylation *in vitro*, which correlates with increased ERK1/2 activation [66]. GRK2 has been shown to negatively regulate the NFκB p105-ERK pathway *in vivo*. GRK2 deletion in myeloid cells of mice increases inflammatory cytokine and chemokine production following LPS stimulation and macrophages derived from these mice show reduced ERK1/2 phosphorylation following LPS and IKK inhibitor treatment [68]. Furthermore, GRK2 may work in concert with GRK5 to

inhibit this pathway, as GRK5 knockout mice only displayed marginally enhanced p105-ERK signalling, suggesting that perhaps GRK2 compensates for loss of GRK5 [69].

Contrasting with the inhibition of p105 by GRK5 following TLR4-stimulation in macrophages, a major role for GRK5 in positively regulating IκBα-NFκB signalling via TLR4 activation has been identified from *in vivo* studies performed by the Parameswaran group. Cytokine and chemokine production was largely attenuated in GRK5 knockout mice and p65 nuclear translocation and IκBα phosphorylation were significantly reduced in LPS treated $GRK5^{-/-}$ macrophages, compared to $GRK5^{+/+}$ macrophages [69]. GRK5 also positively regulates the NFκB pathway invoked following tumour necrosis factor α (TNFα) stimulation, as does GRK2 [70]. Knockdown of GRK2 and GRK5 inhibited IκBα phosphorylation, nuclear translocation of p65/p50 and NFκB transcriptional activity in macrophages following TNFα treatment. Both kinases bind and phosphorylate IκBα, but the sites of phosphorylation are different; GRK5 but not GRK2 phosphorylates IκBα at Ser32/Ser36, the same sites as IKKβ. An IκBα S32A/S36A mutant exhibited approximately 60% reduced *in vitro* GRK5-mediated phosphorylation, compared to wildtype IκBα. GRK2, however, phosphorylated both wildtype and mutant IκBα to the same degree. GRK5 and GRK2 therefore regulate IκBα differently following TNFα stimulation and furthermore, GRK5 regulates IκBα in opposing manners depending on the cell type [65, 70]. Additionally, the positive regulation of NFκB by GRK5 may be evolutionarily conserved. Gprk2, the *Drosophila* equivalent of mammalian GRK5, activates NFκB following bacterial stimulation. This mechanism

is also conserved in zebrafish and in HeLa cells, as knockdown of Gprk2 or GRK5 impaired NFκB transcriptional activity [71].

The regulation of NFκB-mediated gene expression is therefore extremely complex, with the activation of different receptors initiating alternative signalling pathways that involve different methods of NFκB regulation in different cell types. Activation of TLR4 via LPS causes GRK5 to bind and stabilise p105 in macrophages, making the complex less susceptible to IKK phosphorylation, thereby inhibiting NFκB [66]. GRK5 differentially regulates IκBα depending on the stimulus and cell type. LPS induces an interaction between the kinase and IκBα, which stabilises the IκBα/NFκB complex in the nucleus of endothelial cells, thereby preventing NFκB-mediated transcription [65]. In macrophages however, GRK5 positively regulates LPS-induced NFκB signalling by phosphorylating IκBα [69] and a similar mechanism has been shown to occur following TNFR stimulation; TNFα causes GRK5 phosphorylation of IκBα at sites overlapping with IKKβ, thereby relieving NFκB inhibition [70]. The method of GRK5-mediated NFκB regulation therefore varies depending on the stimulus, cell type and IκBα isoform, with GRK5 playing a dual role in regulating NFκB signalling. The implications of the GRK5-mediated NFκB regulation are vast due to the plethora of transcriptional targets of NFκB, including not only cytokines and chemokines but also negative regulators of apoptosis, including B cell lymphoma 2 (Bcl-2), whose role in apoptosis and disease is discussed in sections 1.3.3.2 and 1.4 respectively. Furthermore, deregulation of NFκB signalling is involved in many diseases, with upregulated activity common to colon cancer and pathological cardiac hypertrophy, which are discussed in section 1.4.

1.3.3.2 Apoptosis

DNA damage results in two possible outcomes: delay to cell cycle progression allowing for effective repair, or programmed cell death if the damage is deemed irreparable. Double stranded breaks (DSB) in DNA activate kinases ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR), to trigger a phosphorylation cascade that can result in DNA repair, checkpoint control or apoptosis depending on the extent of the damage. The tumour suppressor protein, p53, is a major determinant of cell fate, whose phosphorylation and stabilisation by ATM controls the switch between apoptosis and growth arrest. The extent of DNA damage affects the sensitivity to p53 levels, which regulates the response to DNA damage by activating different signalling pathways. Following minor DSB only a small fraction of p53 is needed to drive the expression of p21, which halts cell cycle progression by eliciting arrest in G1. This high sensitivity to p53 promotes DNA repair pathways, which would have time to act due to the p53-mediated inhibition of cell growth. In contrast, major DSB result in a higher concentration of p53 accumulating, which in turn leads to the activation of apoptotic pathways [72]. The difference in p53 sensitivity in relation to the severity of DNA damage thereby regulates cell fate.

P53 is crucial in determining the cellular response to stress, not only by inducing apoptosis but by also having a role in growth arrest. Induction of p53-dependent apoptosis occurs via extrinsic or intrinsic pathways depending on apoptosis signals and converges on the activation of caspase proteins (Figure 1.4). Following DNA damage, the intrinsic apoptotic pathway is activated, whereby cytochrome C is released from mitochondria and forms a complex with apoptosis protease-

activating factor 1 (Apaf1). Seven Apaf1 monomers oligomerise in complex with cytochrome C and bind to pro-caspase 9, to form a complex known as the apoptosome. The apoptosome forms a platform for caspase 9 activation, recruiting the pro-caspase 9 zymogen and catalysing its cleavage and thus activation, which in turn triggers a caspase cascade, resulting in the cleavage and activation of effector caspases 3, 6 and 7 [73]. The intrinsic apoptotic pathway is controlled by the Bcl-2 family of proteins, which comprise both pro- and anti-apoptotic members, which regulate the release of cytochrome C from mitochondria. There are three main classes of Bcl-2 proteins: Bcl-2 and its structurally related protein, B cell lymphoma-extra large (Bcl-xl), are anti-apoptotic proteins with a pro-survival phenotype, inhibiting pro-apoptotic Bcl-2 family members, Bax and Bak. Bax and Bak permeabilise the outer mitochondrial membrane, thereby enabling release of cytochrome C, as illustrated in Figure 1.4. All four proteins are structurally similar and contain four Bcl-2 homology (BH) domains. The final group of Bcl-2 family proteins are structurally dissimilar, BH3 only proteins that comprise this BH domain alone and work in a pro-apoptotic manner by binding and inhibiting anti-apoptotic Bcl-2 family members. Examples of BH3 only proteins include Bid, Bad, PUMA and NOXA [74]. Through binding and sequestering Bcl-2 and Bcl-xl, BH3 only proteins relieve the inhibition of pro-apoptotic Bcl-2 family proteins. The extrinsic and intrinsic pathways are linked by the BH3 only protein, Bid, which, as shown in Figure 1.4, is activated by caspase 8, which itself is activated following receptor stimulation that occurs in the extrinsic pathway. Cleavage of Bid exposes an N-terminal glycine residue to myristoylation, which results in its relocalisation to mitochondria where it activates Bax and Bak [73].

P53 triggers the expression of Apaf1 and BH3 proteins and upregulates caspase expression. P53 also upregulates the expression of extrinsic pathway receptors, Fas, DR5 and PERP, by increasing mRNA levels, and promotes trafficking of the Fas receptor to the cell surface from the Golgi. As shown in Figure 1.4, Fas is activated by its ligand, FasL, following tissue specific γ-radiation. The death-domain containing receptor, DR5, is stimulated by its ligand, TRAIL, following DNA damage. PERP is expressed in mouse embryonic fibroblasts (MEFs) but its mechanism of p53-mediated apoptosis is currently unidentified. Initiator caspases 8 and 10 are activated by these receptors and cleave the pro-caspase 3 zymogen, as well as Bid [73].

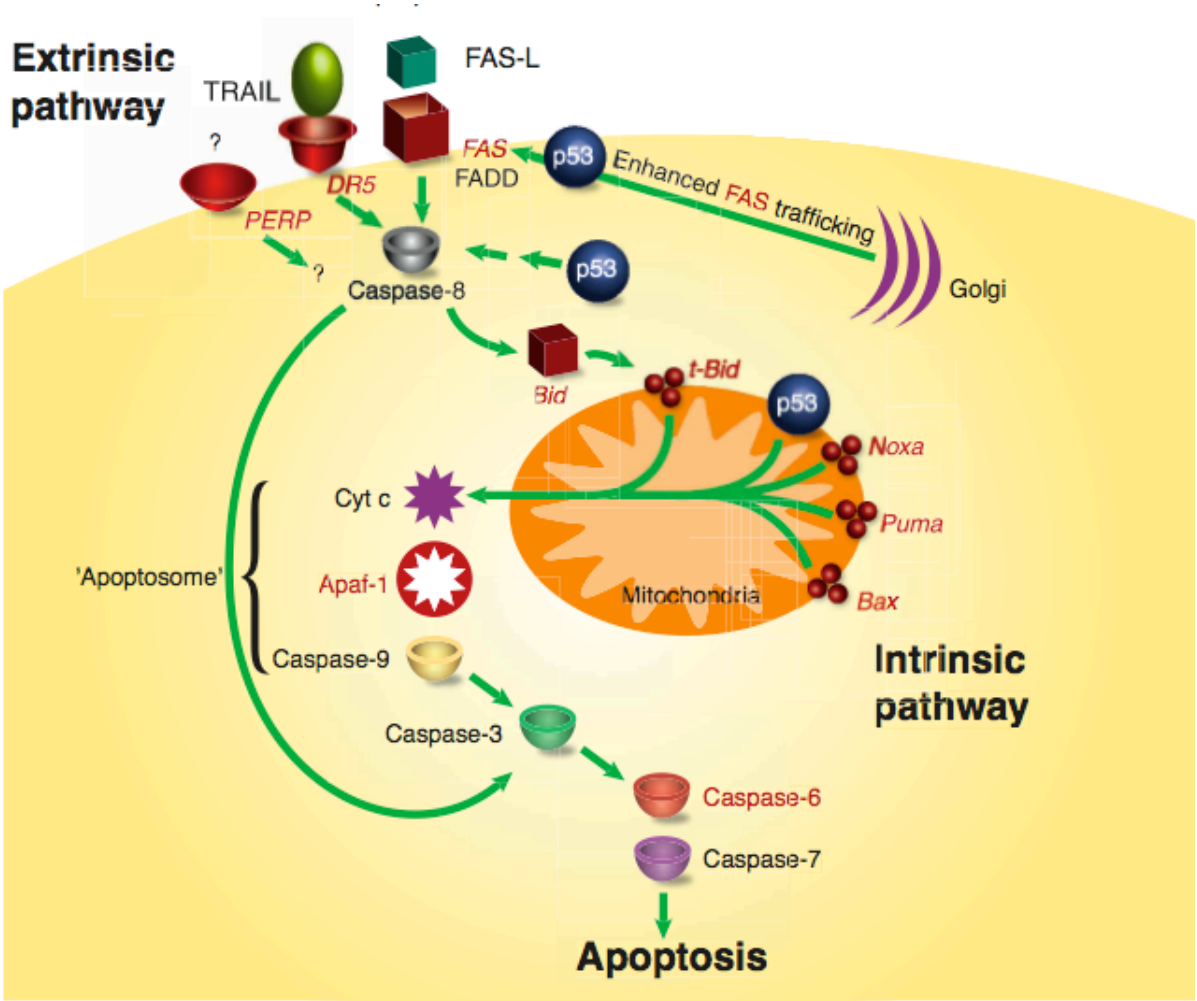


Figure 1.4 Intrinsic and extrinsic apoptosis pathways

DSB are detected namely by ATM and ATR, which activate p53. Once activated, p53 enhances both the intrinsic and extrinsic apoptotic pathways by upregulating the expression of key proteins involved in both pathways, shown in red. P53 can also localise to mitochondria to promote a transcription-independent mechanism of apoptosis via cytochrome C release. Taken from [73].

The Ma group showed GRK5 to phosphorylate p53 *in vitro* and *in vivo* at Thr55, which results in reduced p53 levels. Treatment with the proteasome inhibitor, MG132, prevented the reduction in p53, thus confirming its degradation is via the proteasome. GRK5 knockout mice displayed tissue-wide aberrant upregulation of p53 implying that GRK5 negatively regulates p53 *in vivo.* GRK5-mediated p53 degradation directly affects apoptosis, as knockdown of GRK5 in the osteosarcoma cell line, U2OS, increases apoptosis by 40% following cisplatin treatment. Apoptosis of Saos-2 cells, which are p53 null, were unaffected following GRK5 knockdown, thus suggesting that GRK5 induces apoptosis via p53 [49].

Activation of p53 involves its release from repressors, its stabilisation and DNA binding to trigger the upregulation of target genes. Phosphorylation of p53 is crucial for its stabilisation. N-terminal phosphorylation occurs rapidly after DNA damage and is catalysed by a range of kinases including ATM and ATR. Phosphorylation serves to activate p53 and inhibit the interaction with the E3-ubiquitin ligase, mouse double minute 2 (Mdm2). Mdm2 is the principal ubiquitin ligase that acts on p53, triggering its destruction by the proteasome, but Mdm2-independent pathways of p53 destruction can also occur via other ubiquitin ligases, such as COP1. Phosphorylation of Ser15 and Ser20 are considered most important for activating

p53 and inhibiting Mdm2 binding, yet knockin mouse models with mutated residues Ser18 and Ser23, the mouse equivalent of Ser15 and Ser20, question the importance of these residues. Mutation of either residue to alanine only slightly reduced p53 levels *in vivo* and severe effects in double S18/23A mutant mice were only noted in specific tissues [75]. Either phosphorylation of additional residues is necessary to regulate p53 stability or the mechanism of p53 regulation is far more complex.

Phosphorylation of Thr55 by GRK5 enhances the interaction of p53 with Mdm2 and thus its degradation [49] (Figure 1.2). The interaction between Mdm2 and p53 takes place in the nucleus, as does p53 ubiquitination. Mdm2 promotes the nuclear export of ubiquitinated p53, which leads to its degradation in the cytoplasm [76]. Whether p53 is a nuclear GRK5 substrate is yet to be confirmed but this is highly likely considering the mechanism of p53 regulation. Phosphorylation at Thr55 induces the association of p53 with the nuclear export factor, chromosome region maintenance 1 protein (CRM1) and the promotion of this interaction by Mdm2 is dependent on Thr55 phosphorylation [77]. P53 phosphorylation by GRK5 is therefore likely to occur in the nucleus and may function to catalyse the cytoplasmic shuttling of ubiquitinated p53, to mediate its degradation by the proteasome by enhancing its interaction with Mdm2 and thus CRM1. A functional consequence of Thr55 phosphorylation has been studied in the breast cancer cell line, MCF7; ERK2 phosphorylation of p53 at Thr55 increases sensitivity of cells to the chemotherapeutic drug, doxorubicin (DOXO) [78].

It is worth noting that apoptosis is not solely induced by p53. Over 50% of tumours have mutated p53 proteins, and one of the most common mutations is at Arg273, which renders the protein unable to bind DNA and therefore inactive. While chemotherapy and radiotherapy can induce apoptosis in these cells, potentially via p53 homologues, p63 and p73, or via alternative apoptotic pathways such as NFκB activation, the challenge of inducing apoptosis is much greater if cells are deficient in wildtype p53 [79].

Through its phosphorylation of p53 at Thr55, GRK5 acts to inhibit p53-mediated apoptosis by upregulating p53 degradation via the proteasome. Conversely, GRK5 has been shown to negatively regulate Bcl-2 transcription in SHSY5Y cells, which is predicted to increase cell death, although the impact of this regulation on apoptosis has not been examined prior to my thesis work [80]. Deregulated apoptosis plays a key role in many diseases. In cancer, p53 is often mutated and apoptosis is downregulated in order to promote unprecedented cell growth. Conversely, in cardiovascular diseases, apoptosis is often prevalent, causing cardiac myocyte cell death. GRK5 expression levels vary in disease states including cancer and cardiac hypertrophy and may thereby differentially regulate apoptosis. The contribution of apoptosis and GRK5 to disease progression, including cancer and pathological cardiac hypertrophy, will be discussed in more detail in section 1.4.

1.3.3.3 Histone deacetylase (HDAC) 5 phosphorylation

Subjecting mice to cardiomyocyte-specific overexpression of wildtype GRK5 and inducing hypertrophy via transverse aortic constriction (TAC), results in

exaggerated pathological hypertrophy, premature heart failure and death. Mice overexpressing GRK5ΔNLS, however, behave like control littermates [81]. The elucidation of a mechanism whereby nuclear GRK5 causes hypertrophy led to the identification of the first nuclear substrate of the kinase, HDAC5.

Nuclear activation of the transcription factor, myocyte enhancing factor 2 (MEF2), downstream of Gq-GPCRs, involves the regulation of class II HDACs, of which HDAC5 is a member. HDAC5 binds and sequesters MEF2 in the nucleus under basal conditions. Phosphorylation of HDAC5 triggers its release of MEF2 and its concurrent nuclear export via the recruitment of 14-3-3 proteins. MEF2 is subsequently able to activate pro-hypertrophic foetal cardiac genes, which are aberrantly expressed in pathological hypertrophy [82, 83], such as atrial natriuretic factor (ANF) whose transcriptional upregulation is illustrated in Figure 1.2. This cardiac remodelling, switching from the adult to foetal gene program, occurs following various stress triggers including volume or pressure overload, myocardial infarction and hypertension. By identifying HDAC5 as a GRK5 substrate, the Koch group identified the first nuclear function of GRK5. GRK5 was identified as a novel HDAC5 kinase, phosphorylating HDAC5 at Ser259 and Ser498, the same sites targeted by previously identified HDAC5 kinases, CaM kinase II (CaMKII) and protein kinase D (PKD) [81, 83, 84]. GRK5 binds HDAC5 in NRVM and phosphorylates HDAC5 *in vitro*. The kinase activity of GRK5, as well as its nuclear localisation is therefore important for the resultant upregulation of MEF2 activity [81]. The implications of this mechanism of GRK5-mediated pathological cardiac hypertrophy will be discussed in more detail in section 1.4.1, where the role of GRK5 in cardiovascular disease is explained in depth.

1.4 GRK5 in disease

The altered expression of GRKs is associated with many disease states including cardiovascular disease (CVD), cancer and psychiatric disorders.

1.4.1 Pathological cardiac hypertrophy

GRK5 and GRK2 are the primary GRK members expressed in the heart. A hallmark of human heart failure is impaired β-adrenergic receptor signalling. GRK2 is the predominant kinase responsible for β-adrenergic receptor phosphorylation, such that GRK2 upregulation and increased activity have long been known to play a causative role in CVDs, particularly hypertension [85, 86]. GRK5 is upregulated in patients with left ventricular overload disease, which is associated with hypertrophic cardiomyopathy (HCM) but the role of GRK5 in cardiac myocyte signalling has been less understood until recently [87, 88]. The significance of elevated GRK5 levels were only appreciated when mice with cardiac specific overexpression of GRK5 were stressed by TAC during experiments performed by the Koch group to investigate whether elevated GRK5 levels influenced the myocardial response to pressure overload and affected the development of pathological hypertrophy [81]. The murine TAC model mimics pressure overload and is used extensively as a tool to model human CVD and to probe the mechanism behind cardiac hypertrophy and the development of heart failure *in vivo* [89]. Patients with ventricular overload disease have elevated cardiac expression of GRK5, such that transgenic mice overexpressing GRK5 are predicted to model the disease [88, 90]. It was only by stressing the transgenic mice that the contribution of GRK5 to pathogenesis was understood. Compared to littermate control mice and GRK2 transgenic mice that

behaved in a similar manner, subjecting mice to cardiac myocyte-specific overexpression of wildtype GRK5 resulted in exaggerated pathological hypertrophy, premature heart failure and death in response to TAC. This response was abrogated in GRK5ΔNLS transgenic mice, implicating nuclear GRK5 as a causative factor in disease pathology. Investigating the mechanism in NRVM, the Koch group found GRK5 to accumulate in nuclei in response to hypertrophic stimuli, such as overexpression of Gq coupled GPCRs. A potential mechanism by which nuclear GRK5 exaggerates hypertrophy development is by acting as an HDAC5 kinase, whose phosphorylation triggers its nuclear export, relieving the inhibition of MEF2 and thus resulting in the upregulation of pro-hypertrophic foetal cardiac genes [81]. These data therefore implicate GRK5 in a pro-hypertrophic mechanism, suggesting a causative role for the kinase in progression towards heart failure.

The Iaccarino group has shown an N-terminal GRK5 peptide to be cardioprotective *in vitro* and *in vivo*. As previously discussed, the N-terminal domain of GRK5 inhibits NFκB mediated transcription, as the RH domain interacts with inhibitory subunit, IκBα, preventing its phosphorylation and subsequent degradation in endothelial cells [65]. NFκB activity is activated in human failing hearts and *in vitro* studies have demonstrate the requirement of this transcription factor for hypertrophic growth of cardiac myocytes in response to GPCR agonists [91, 92]. *In vivo* and *in vitro* expression of an adenovirus that encodes for the RH domain within the amino terminal of GRK5 (AdGRK5-NT) prevented left ventricular hypertrophy [93]. These data therefore suggest that the N-terminus of GRK5 functions in an anti-hypertrophic manner, in contrast to the role of nuclear GRK5 as an HDAC5 kinase, which has a pro-hypertrophic function. The effect of the intact

kinase on NFκB-mediated cardiac hypertrophy, however, has not been studied. Since mice lacking endogenous cardiac GRK5 are protected from TAC-induced hypertrophy, I predict GRK5 to be acting in a dominant pro-hypertrophic manner.

GRK5 is further implicated in hypertrophy, potentially via its canonical role in receptor desensitisation. A cardioprotective GRK5 Gln41Leu polymorphism has been found among the African American community [14]. The Liggett group attributed the beneficial effects mediated by the Leu41 polymorphism to augmentation of β-adrenergic receptor desensitisation, based on *in vitro* studies and on transfected cells and transgenic mice overexpressing the Gln41Leu GRK5 mutant [94, 95]. A study in humans, however, contests this theory and showed the GRK5 polymorphism did not affect sensitivity to the β-adrenergic receptor blocker, atenolol, following acute adrenergic stimulation [96]. This discrepancy may be due to the acute stimulus used in the human study, as prolonged adrenergic stimulation is more likely to enhance receptor desensitisation.

The role of GRK5 in pathological cardiac hypertrophy is therefore extremely complex. While the N-terminal RH domain of GRK5 appears to have a cardioprotective function, the intact kinase exacerbates pathological cardiac hypertrophy. Upregulation of GRK2 occurs to a greater degree than GRK5 in heart failure patients and, as such, the physiological role and regulation of GRK5 in cardiovascular diseases has not been extensively characterised. Studies by the Koch group have greatly advanced the appreciation of GRK5 as a causative component of pathological cardiac hypertrophy. Elucidation of the mechanisms regulating GRK5 nuclear localisation and activity may thus lead to important

therapeutic advances.

1.4.2 Neurodegenerative disease

Alzheimer's disease (AD) represents one of the most common forms of dementia. Most patients with Parkinson's disease (PD) develop dementia in later stages of the disease, but the hallmark of PD is degeneration of the central nervous system. GRK5 has been implicated in the pathology of both of these neurodegenerative diseases.

AD is a neurodegenerative disease pathologically associated with the accumulation of β-amyloid plaques and neurofibrillary tangles, which are composed of phosphorylated tau, in neurons. Imbalanced neuronal signal transduction is a hallmark of the disease and is attributed to hyperactive G protein signalling, which can arise due to impaired GPCR desensitisation. In the context of AD, perturbed receptor desensitisation potentially results from β-amyloid-induced relocalisation of GRK5 to the cytoplasm. Pre-treatment of microglia with β-amyloid enhanced thrombin induced GPCR activity and significantly reduced interactions of GRK5 with the GPCR, PAR1. While thrombin treatment stimulated the GRK5/PAR1 interaction, as would be expected following GPCR activation, pre-treatment with β-amyloid reduced the binding of GRK5 by approximately 80%. Immunofluorescence experiments attributed the loss of GRK5 binding to GPCRs to the β-amyloid stimulated cytoplasmic re-localisation of GRK5. *In vivo* work using AD transgenic mice showed this relocalisation occurs at the early stages of disease pathogenesis, prior to cognitive decline and persists into the later stages of the disease [97]. This suggests that loss of GRK5 at the membrane may be a causative factor,

contributing to AD pathogenesis. Aged GRK5 knockout mice displayed mild cognitive impairment, with short-term memory deficiencies and hypoalertness, as well as increased axonal defects. Clusters of axonal swellings were also noted, which included elevated levels of total and phosphorylated tau, as well as β-amyloid [98].

The mechanism by which GRK5 plays a causative role in AD is unclear but the GRK5 deficiency at the plasma membrane links both the cholinergic and β-amyloid hypotheses of AD pathogenesis. These hypotheses attribute the cognitive decline associated with AD to β-amyloid accumulation, which is toxic to neurons, or deregulated cholinergic signalling, respectively [99]. Presynaptic hypercholinergic dysfunction reduces acetylcholine release resulting in post-synaptic hypocholinergic signalling, both of which are detected in cortex and hippocampal tissues of AD patients, along with a reduced activity of choline acetyltransferase, which catalyses acetylcholine production [100]. Instead of conflicting, the two hypotheses rather emphasise different aspects of disease pathology and are likely to integrate to some degree. Indeed, post-synaptic hypocholinergic activity promotes the processing of the β-amyloid precursor protein, which thus promotes β-amyloid accumulation. Furthermore, β-amyloid is the main cause of GRK5 cytoplasmic relocalisation and it is the relocalisation of the kinase that impairs cholinergic desensitisation [99].

PD pathology involves the degeneration of dopaminergic neurons in the substantia nigra and the accumulation of phosphorylated α-synuclein proteins in Lewy bodies, which are neuronally toxic. Synucleins are soluble proteins expressed primarily in

neurons and no discernable function has been identified for either α-, β- or γ-synuclein. Via interactions with tubulin, there is potential for α-synuclein to be a microtubule associated protein and it may play a role in blocking endoplasmic reticulum-to-Golgi trafficking in PD [45, 101]. GRK5 co-localises with α-synuclein in neurons of PD patients, accumulating in Lewy bodies where it functions as an α-synuclein kinase. Overexpression of GRK5 in HEK293 cells stably expressing α-synuclein and treated with a phosphatase inhibitor resulted in significant phosphorylation of α-synuclein at Ser129, which increased with increasing concentration of OA, a protein phosphatase 1/2A inhibitor. In the *Drosophila* model of PD, phosphorylation of α-synuclein at Ser129 renders the protein toxic to dopaminergic neurons, potentially by inducing the aggregation of α-synuclein monomers [102, 103]. The Qin group in China, however, showed that knockdown of GRK5 in the neuroblastoma cell line, SHSY5Y, did not affect Ser129 phosphorylation levels [80]. This latter finding implies that while α-synuclein is a GRK5 substrate, GRK5 is not the sole kinase involved in catalysing Ser129 phosphorylation and suggests that other kinases can compensate for the loss of GRK5. The group went on to show that GRK5 expression levels are upregulated in α-synuclein stably expressing mice and that the kinase upregulates HDAC activity in these cells compared to SHSY5Y cells. α-Synuclein stably expressing cells, which have higher GRK5 expression levels compared to SHSY5Y cells, also have lower Bcl-2 protein levels and the same is true for α-synuclein stably expressing mice compared to control, nontransgenic mice. Reverse transcription – polymerase chain reaction (RT-PCR) experiments showed knockdown of GRK5 in SHSY5Y cells to slightly but significantly increase Bcl-2 mRNA levels [80]. Additionally, post-mortem studies illustrated that PD patients have increased GRK5 protein and

mRNA levels [104]. So while GRK5 may not have a crucial role as an α-synuclein kinase, upregulation of GRK5 in PD is likely to play a causative role in pathogenesis, potentially by inducing transcriptional repression of Bcl-2 by upregulating HDAC activity, whose function is discussed in detail in section 1.5. The consequence of GRK5-mediated Bcl-2 inhibition on PD progression is currently uncertain, but may suggest a role for apoptosis in disease pathology.

1.4.3 Cancer

Aberrant activation and/or expression of GPCRs are associated with many malignancies, which thereby implicates GRKs in cancer pathology. Unprecedented cell growth and proliferation, angiogenesis and suppression of apoptosis are common cellular features of cancer cells. The role of GRK5 in cell cycle regulation and apoptosis, as discussed in section 1.3.2.1 and 1.3.3.2, respectively, implicates GRK5 in cancer pathogenesis beyond its role as a GPCR kinase. Moreover, findings by the Qin group that GRK5 upregulates HDAC activity, suggests an additional function for GRK5 in promoting gene repression and considering cancer is associated with aberrant gene expression, reinforces a potential role for GRK5 in this collection of diseases, which is the main focus of my thesis.

1.4.3.1 Prostate cancer

GRK5 has been directly implicated in prostate cancer pathogenesis, as knockdown of the kinase in the prostate cancer cell line, PC3, attenuated proliferation. Stable knockdown of GRK5 using a shRNA construct also reduced tumour growth rates following the implantation of these cells into immunodeficient mice. A kinase-

dependent function of GRK5 in regulating prostate tumour growth is implied, as tumour growth rates were restored following the overexpression of wildtype but not kinase dead GRK5 and kinase activity is important in regulating cell cycle progression of PC3 cells [12].

GRK5 knockdown in PC3 cells caused an accumulation of cells in G2/M, with a concomitant decrease of cells in G1 phase of the cell cycle. Consistent with these findings, levels of the cell cycle markers, cyclin D1 (G1-phase marker) and phosphorylated histone H3 (G2/M marker), were reduced and elevated respectively [12]. As explained in section 1.3.2.1, GRK5 is implicated in cell cycle regulation via interactions with tubulin, its localisation at centrosomes and its promotion of centrosome nucleation. GRK5 knockdown in HeLa cells leads to G2/M cell cycle arrest [48] and a similar finding in PC3 cells reinforces the role played by GRK5 not only in cancer pathogenesis but also in cell cycle regulation. A possible mechanistic explanation of GRK5-mediated regulation of prostate cancer cell proliferation is via the phosphorylation of retinoblastoma protein (Rb). Rb hyperphosphorylation is detected in the early stages of G1 in rapidly proliferating cancer cells, as the phosphorylated protein relieves inhibition of the transcription factor, E2F1. Knockdown of GRK5 resulted in a hypophosphorylated form of Rb, and abrogated expression of E2F1 target genes [12]. The role of GRK5 in regulating cell cycle progression thereby highlights a role for GRK5 in prostate cancer pathology.

1.4.3.2 Glioblastoma multiforme (GBM)

GRK5 has also been implicated in the proliferation of GBMs, which are the most common brain tumours in adults. These tumours are thought to arise from GBM initiating cells with stem cell markers (GSC). A role for GRK5 in GBM has been elucidated, with expression levels of the kinase being highest in primary GBMs and positively correlating with the aggressiveness of the tumour. Furthermore, GRK5 is more highly expressed in GSC, which, compared to non-GSC or differentiated GBM cells, are more resistant to treatment [105]. While the cause and mechanistic implications of altered GRK5 expression are yet to be elucidated, GRK5 has been shown to regulate GBM proliferation. Stable knockdown of GRK5 in GBM cells significantly reduced proliferation rates [105].

1.4.3.3 Thyroid carcinoma

GRK5 and GRK2 are the predominant GRK isoforms in human thyroid, playing a role in regulating thyroid stimulating hormone (TSH) receptors [106, 107]. TSH receptor stimulation is linked to elevated levels of cyclic AMP (cAMP) and basal levels of cAMP have been found to be significantly higher in differentiated thyroid carcinoma (DTC) compared to normal thyroid tissue (NTT) [108, 109]. Reduced GRK5-mediated TSH desensitisation is thought to play a role in disease pathogenesis.

Despite the involvement of both kinases in TSH receptor desensitisation, expression of GRK5 but not GRK2 was significantly reduced in DTC in comparison to NTT. In contrast, rhodopsin-directed GRK activity was upregulated in DTC [110].

The enhanced TSH activity could be attributed to the reduced GRK5 levels, while the increased GRK activity is likely to be GRK2 dependent. All samples assayed for GRK activity were tested for GRK2 protein expression levels and while no significant difference in GRK2 expression levels were detected overall by Western blotting, two patients out of the ten had paired samples showing relatively higher GRK2 protein expression compared to their normal tissues. A positive correlation was observed for GRK2 expression levels and GRK activity and considering rhodopsin is a preferred substrate of GRK2 compared to GRK5, GRK2 upregulation is the likely cause of the increased GRK activity [110].

TSH downregulates GRK5 protein and mRNA levels in rat thyroid cells and the reduced TSH desensitisation in DTC compared to NTT suggests that GRK5 may also regulate receptor signalling [106, 110]. GRK5 expression may therefore account for the perturbed TSH receptor desensitisation and subsequent upregulation of cAMP levels in thyroid tumours.

1.4.3.4 Colorectal cancer (CRC)

Considering CRC is a main focal point of my thesis, I will explain the disease pathogenesis in more detail compared to the other diseases mentioned thus far. CRC is the second largest cause of cancer mortality in the United States; it is estimated that more than 100,000 new cases were diagnosed in 2010 alone, half of which resulted in death [111]. Patient prognosis correlates with the stage at which the disease is detected; survival of patients five years post diagnosis ranges from 74–93.2% for those diagnosed with stage I cancers, which are confined to the colon, but is only 5.7–8.1% for those with stage IV, metastatic disease [112]. It is

estimated that 60% of CRC related deaths could be prevented if screening methods were more widely utilised [113]. Stage I cancers can be cured by aggressive surgery, yet only 39% of patients present with such tumours at the time of diagnosis [American Cancer Society, *Cancer Facts and Figures*, 2012]. Once the cancer spreads beyond the inner lining of the colon and metastasises to the lymph nodes, patients can be treated post surgery with adjuvant chemotherapy agents such as 5-fluorouracil (5-FU), which acts as the backbone in most combinatorial therapeutic approaches. One combinatorial therapeutic study in stage II and III CRC patients treated with 5-FU, leucovorin and oxaliplatin resulted in a 78.2% survival rate three years post treatment [114]. Treatment of stage IV metastatic CRCs represents a more serious clinical problem particularly if secondary tumours are not resectable, as such late stage cancers are often chemo-resistant.

Over 50% of CRCs arise from large, pre-existing adenomas, or polyps, whose benign cells undergo transformation over a series of years and thus become malignant [115]. Transformation ensues following the accumulation of four to seven genetic mutations that result in altered cellular proliferation. 60–80% of adenomatous polyps show loss of heterozygosity in the APC gene, which is believed to be one of the earliest acquired mutations in the development of CRC [115, 116]. A collection of mutations in key oncogenes and tumour suppressor genes including MLH-1, K-Ras and p53, arise in a non-linear fashion, causing the adenomatous polyp to progress into a carcinoma. Additionally, overexpression of the anti-apoptotic protein, Bcl-2, is associated with early development of CRCs [116]. Bcl-2 is a key regulator of the intrinsic apoptotic pathway, such that its

overexpression may, at least in part, be responsible for apoptosis resistance, the hallmark of cancerous cells.

A whole genome association study, published in a patent application by Dr Stephen Gruber at the University of Michigan, identified a single nucleotide polymorphism (SNP) in the intron of GRK5 to be associated with a 35% increased risk of developing CRC [Patent publication number: WO2009046422 A2]. It is currently unknown whether this SNP affects GRK5 expression levels, but a recent microarray study of gene expression profiles of patients with colorectal liver metastases identified GRK5 as being significantly under expressed in higher-risk patients [Chung, J., 2013; personal communication]. There is, therefore, potential for GRK5 to act as a biomarker for CRC, with levels of the kinase possibly indicating the stage of disease. Moreover, GRK5 has also been shown to negatively regulate Bcl-2 transcription in SHSY5Y cells, levels of which, as previously mentioned, are upregulated in colon cancer [80]. It therefore feasible that GRK5 may be playing a causative role in colon cancer progression to a metastatic state; down-regulation of the kinase may relieve Bcl-2 transcriptional inhibition and render cells resistant to pro-apoptotic stimuli, thus accelerating cancerous cell growth.

Chapter 5 of my thesis explains a potential mechanism whereby GRK5 may increase the sensitivity of the colon cancer cell line, HT-29, to apoptosis induced by 5-FU in an HDAC-dependent manner.

1.5 HDACs and repressor complexes

HDACs are key to the regulation of gene expression and act characteristically to silence genes while their functional counterparts, histone acetyltransferases (HATs), promote transcription [117]. These repressor proteins were first identified in yeast, as HDAC complexes, HDA and HDB. Further characterisation identified two related transcriptional regulators known as Hda1p and Rpd3p, which are responsible for the deacetylation function that correlates with heterochromatin and genetically 'silenced' genes [118, 119]. Control of gene expression by HATs and HDACs is via the addition and removal of acetyl groups. HATs catalyse the acetylation of lysine residues on histone carboxyl terminal tails, which reduces the strength of the electrostatic interaction between the histone and DNA in the nucleosome unit. The resulting chromatin relaxation accommodates transcriptional machinery, thus promoting gene expression. The removal of acetyl groups by HDACs condenses the chromatin and therefore promotes transcriptional repression [120].

A total of eighteen HDACs have been identified in mammals, which are categorised into four classes based on structure and homology to equivalent enzymes in yeast. The yeast HDACs, Hda1p and Rpd3p, represent archetypal members of classical mammalian class I and class II HDAC families, which comprise HDACs 1, 2, 3, 8, and 4, 5, 6, 7, 9, 10 respectively. These HDACs share a conserved catalytic, deacetylase domain, as does the class IV HDAC, HDAC11 (Figure 1.5). Class III HDACs, also known as sirtuins, share no homology with the other HDAC classes, as their deacetylase activity depends on NAD^+ as opposed to zinc ions [117]. Class I, II and IV HDACs catalyse histone deacetylation via a charge-relay system that is

mediated via conserved residues in the catalytic domain and is coordinated by zinc ions [117].

Class I and II are known as classical HDACs and are depicted in Figure 1.5. Class I HDACs are ubiquitously expressed in mammalian cells, while class II HDACs, which are further divided into class IIa (HDACs 4, 5, 7, 9) and IIb (HDACs 6 and 10), are considered to have more specialised functions, as they are expressed only in the brain, skeletal muscle and heart [117]. Class I HDACs have a predominant nuclear localisation whereas class II are subject to stimulus-induced cytoplasmic-nuclear shuttling [121]. The nuclear export of HDACs 4, 5 and 7 to the cytoplasm occurs during muscle differentiation, following phosphorylation by CaMK [117]. Phosphorylation is a key method of HDAC regulation and while both class I and class II are subject to this post-translational modification, phosphorylation controls different aspects of HDAC function. Whereas phosphorylation of class II HDACs regulates their cellular localisation, which indirectly affects their enzymatic activity, class I HDAC phosphorylation directly promotes their deacetylase function, except for HDAC8, which is inhibited by phosphorylation. Furthermore, the extent of class I HDAC phosphorylation provides an additional method of regulation, with HDAC1 and HDAC2 existing in three different phosphorylation states: hypophosphorylated, basally phosphorylated and hyperphosphorylated. In addition to phosphorylation, both classes of classical HDACs are also regulated by sumoylation and polyubiquitination, with HDAC2 also modified by s-nitrosylation and HDAC1 by acetylation [121, 122]. HDAC regulation is further controlled at the transcriptional level, as well as via the association with other transcriptional proteins. Furthermore,

class I HDACs are often found in large multiprotein complexes, which bring specificity to their deacetylase function and mediate their localisation to DNA.

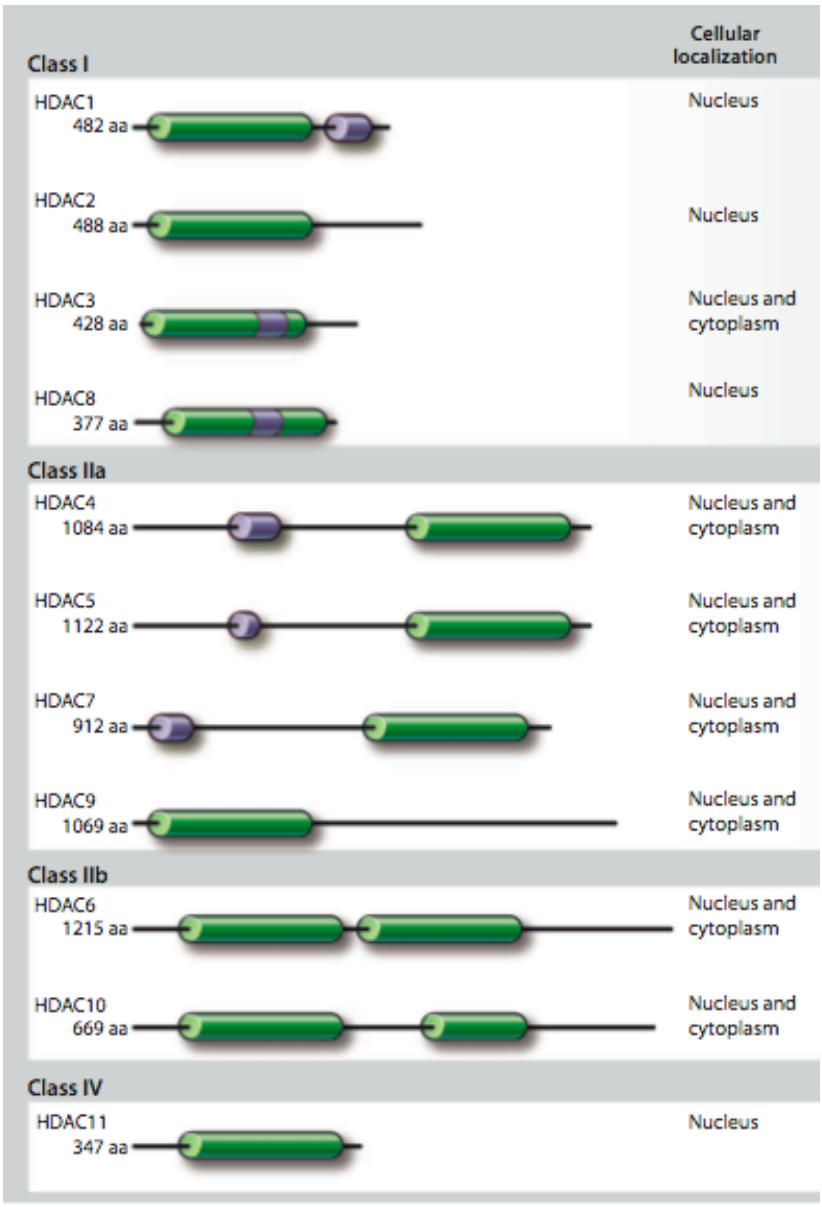
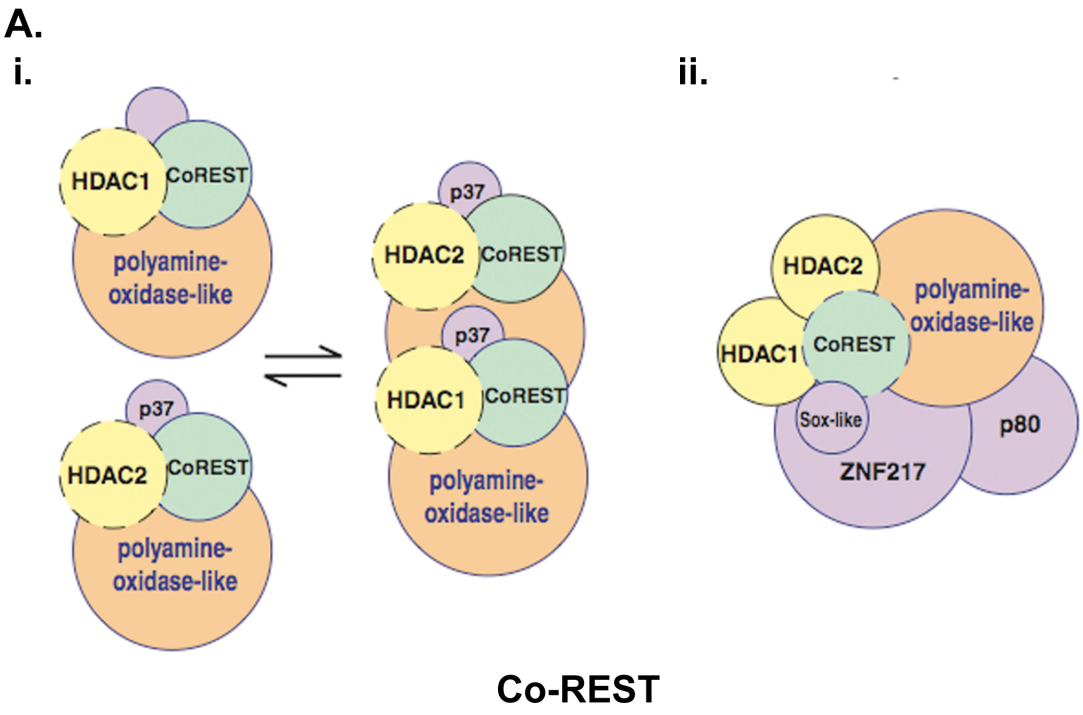


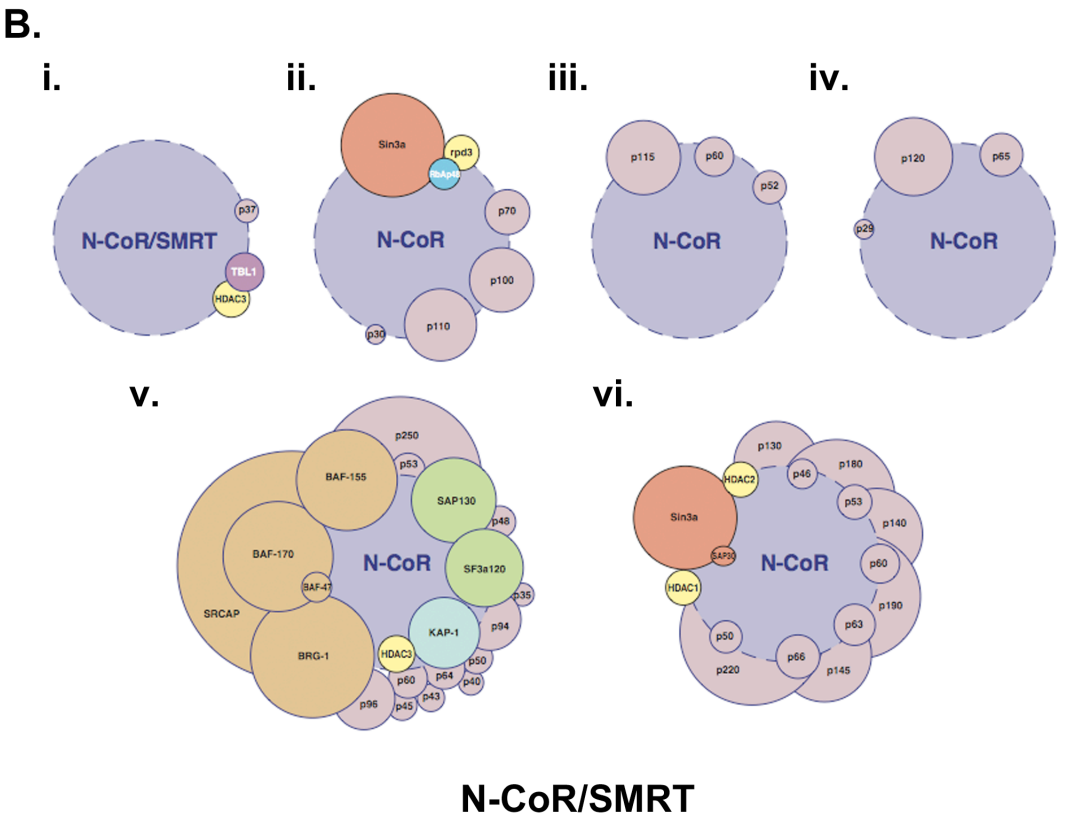
Figure 1.5 Class I and class II HDACs

Organisation of HDAC subfamilies is illustrated in the schematic. Catalytic domains are coloured in green and nuclear localisation signals in purple. HDAC cellular localisation is also given. Taken from [121].

Class I and II HDACs have been implicated in many diseases including CVD and cancer. Pan-HDAC inhibitors that reversibly block HDAC activity, including trichostatin A (TSA) and sodium butyrate (NaB), are often used in research and two such inhibitors, SAHA and depsipeptide, are FDA approved as anti-cancer treatments. Both class I and II HDAC members are upregulated in different cancers, yet class I-specific HDAC inhibitors are considered by many to be the most relevant anti-cancer targets due to their potency in inhibiting proliferation and inducing apoptosis [123]. The class I HDAC inhibitor, MS-275, has been used to treat patients with leukaemia, lymphoma or solid tumours in phase I and II clinical trials [124]. Treatment with TSA suppresses *in vitro* and *in vivo* models of cardiac hypertrophy [125, 126]. HDACs are considered important integrators of the divergent stress response signalling pathways that are activated during cardiac remodelling in response to hypertrophic stimuli [127]. Class IIa HDACs are the best-characterised class in terms of their role in hypertrophy, with HDAC5 acting in an anti-hypertrophic manner by physically inhibiting MEF2. The cardioprotective effect of TSA treatment therefore presents a paradox in terms of HDAC contribution to cardiac hypertrophy, which has been reconciled by opposing functions attributed to class I and class II HDACs. While class II HDACs are anti-hypertrophic, class I are pro-hypertrophic. TSA thereby inhibits class I pro-hypertrophic activity and the anti-hypertrophic effects of HDAC5 remain unaffected, as they are independent of deacetylase action [128]. Indeed, following hypertrophic induction, disease development was significantly reduced in mice treated with the class I specific HDAC inhibitor, SK-7041 [129].

Class I HDACs rarely function independently, but instead work as part of a repressor complex, the association of which acts as another method of regulating HDAC enzymatic activity. Indeed, HDAC1 and HDAC2 only display maximal catalytic activity when involved in a repressor complex [117, 130]. Moreover, the distinct roles played by HATs and HDACs are now considered to be an oversimplification of transcriptional regulation *in vivo* and instead, the outcome may depend on the repressor complex involved [131]. A variety of mammalian repressors have been characterised to recruit both of these HDACs: RE1 silencing transcription factor (REST), which is also known as neural restricted silencing factor and its co-repressor, Co-REST, nuclear receptor co-repressor (N-CoR) and silencing mediator of retinoic and thyroid hormone receptors (SMRT), nucleosome remodelling and deacetylating complex (NuRD) and the mammalian Swi-independent 3 (Sin3) protein [130, 132-134]. These repressors have overlapping as well as distinct binding partners, to coordinate different methods of gene repression, including ATP-dependent chromatin remodelling, histone deacetylation and DNA methylation. Figure 1.6 illustrates the multiple complexes formed by each of these repressors.


A.
i.
ii.
HDAC1
CoREST
polyamine-oxidase-like
p37
HDAC2
CoREST
polyamine-oxidase-like
HDAC2
polyamine-oxidase-like
HDAC1
CoREST
Sox-like
p80
ZNF217
Co-REST


B.
i.
ii.
iii.
iv.
N-CoR/SMRT
p37
TBL1
HDAC3
Sin3a
rpd3
RbAp48
N-CoR
p70
p100
p110
p30
p115
p60
p52
N-CoR
p120
p65
p29
N-CoR
v.
vi.
p250
BAF-155
p53
SAP130
p48
BAF-170
N-CoR
SF3a120
p35
BAF-47
SRCAP
KAP-1
p94
BRG-1
HDAC3
p50
p60
p64
p40
p43
p96
p45
p130
HDAC2
p46
p180
Sin3a
p53
p140
SAP30
N-CoR
p60
HDAC1
p190
p50
p63
p66
p220
p145
N-CoR/SMRT

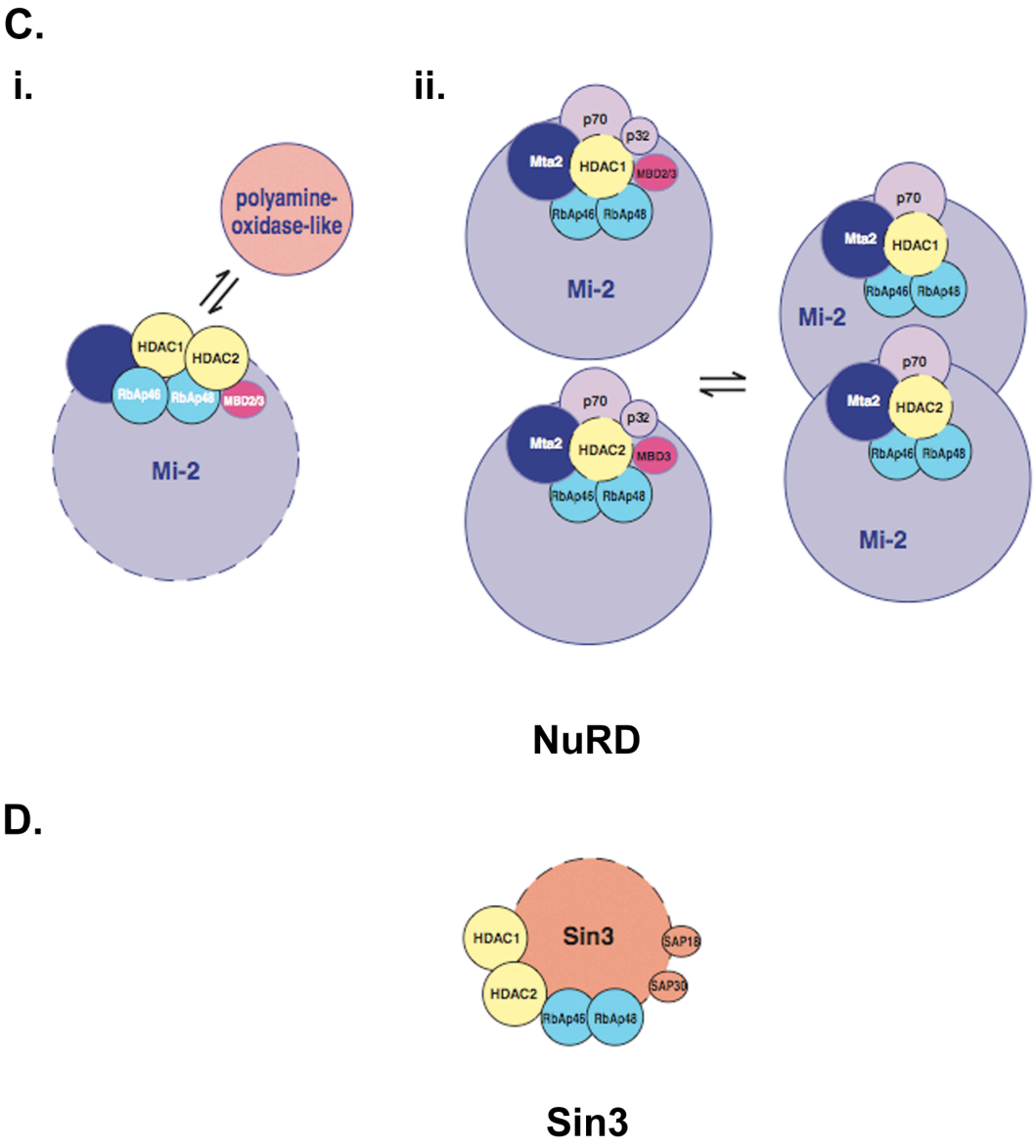


Figure 1.6 Mammalian repressor complexes

The four mammalian transcriptional repressor complexes and their associated proteins. Taken from [90]. **A.** Co-REST complex, currently identified as two distinguishable Co-REST complexes, each of which contains a polyamie-oxidase-like protein as well as HDAC1 and/or HDAC2. **i.** Two interchangable Co-REST complexes have been identified, comprising either HDAC1 or HDAC2, as well as p37, which are able to dimerise [135]. **ii.** Co-REST and polyamie-oxidase-like protein have also been purified with proteins ZNF217, a Sox-like protein and p80 [132]. **B.** N-CoR/SMRT complex, currently purified as six different macromolecule complexes identified

by three separate groups. **i.** Identified from HeLa cells, one N-CoR/SMRT complex contains the transducin β-like protein 1 (TBL1), HDAC3 and a protein of 37 kDa, p37 [136]. **ii. iii. iv.** Three further N-CoR/SMRT complexes were identified from *Xenopus* egg extracts. **ii.** The canonical mechanism of N-CoR/SMRT-mediated repression is reliant on Sin3A, its associated protein, RbAp48 (RBBP4) and HDAC1 (Rpd3). Additional proteins in the complex include p110, p30, p100 and p70 **iii.** A Sin3A-independent N-CoR/SMRT complex has also been identified to comprise p115, p60 and p52. **iv.** A N-CoR/SMRT complex comprising p120, p65 and p29 does not posses HDAC activity [137]. **C.** The NuRD repressor complex comprises HDAC1 and 2, along with RbAp46 and 48, Mi-2, MTA1-3 proteins and MBD2/3. **i.** The potential association of the NuRD complex with polyamine-oxygenase-like protein suggests a potential role for the NuRD complex in redox reactions [138]. **ii.** Differential Mi-2 complexes may also exist, similar to Co-REST, involving either HDAC1 or HDAC2 monomers, which can subsequently dimerise depending on the association with MBD2 [135]. **D.** The Sin3 core complex comprises HDACs 1 and 2 along with Rb-associated proteins, RbAp48 and RbAp46, SAP30 and SAP18 [139].

1.5.1 REST/Co-REST

The transcription factor, REST, comprises eight zinc fingers and is composed of two domains. The N-terminus recruits the Sin3 complex, which helps mediate gene repression, while the C-terminus binds to its co-repressor, Co-REST. [140]. The complex thereby regulates transcription via two distinct mechanisms.

Co-REST comprises two 50-amino acid SANT domains, named for their presence in Swi3, Ada2, N-CoR, and TFIIB. The N-terminal SANT domain is responsible for binding HDACs and is therefore important for repressor activity [90, 132]. Co-REST binds HDAC1 and HDAC2 in a complex distinct from REST and Sin3, as shown in Figure 1.6A [132]. Two major HDAC1/2-containing Co-REST complexes were identified in HeLa cells, both containing the transcriptional co-repressor, CoREST,

a protein homologous to a polyamine-oxidase, which is an FAD-dependent enzyme, as well as a protein of 37 kDa (p37) of unknown function. Under native conditions, these complexes, as shown in Figure 1.6Ai, may contain either HDAC1, or HDAC2. A general model for interaction between HDAC1 and HDAC2 is suggested via dimerisation [135].

Immunoprecipitation of Co-REST from T-Ag Jurkat cells detected both HDACs as interactors of Co-REST, but instead of detecting an interaction with REST, another eight zinc-fingered transcription factor, termed ZNF217, was identified. As shown in Figure 1.6Aii, this Co-REST complex also comprises a 40 kDa Sox-like protein, the polyamine-oxidase-like protein, and p80, a protein of unknown function [132].

1.5.2 SMRT/N-CoR

N-CoR and SMRT are homologous proteins, identified by their association with unliganded retinoic acid (RAR) and thyroid hormone (T_3R) nuclear receptors [141, 142]. RAR- and T_3R-mediated repression occurs independently of agonist binding, with the assistance of N-CoR and SMRT, which have preferential action towards T_3R and RAR respectively [143]. Aside from this difference, N-CoR and SMRT are known to have homologous functions and will, therefore, subsequently be referred to as N-CoR/SMRT.

The mechanism of N-CoR/SMRT repression is most commonly reliant upon Sin3 and HDAC1, but the proteins can also interact directly with HDAC3, HDAC4, HDAC5 and HDAC7 and form a variety of other complexes, as illustrated in Figure 1.6B. Indeed, multiple N-Co-R/SMRT complexes have been identified. A novel N-

CoR/SMRT-containing complex, isolated from HeLa cells, incorporates the transducin β-like protein 1 (TBL1), as well as HDAC3 and another protein of 37 kDa, p37 (Figure 1.6Bi) [136]. Three distinguishable macromolecular complexes were identified in 2001 from *Xenopus* egg extract; one contains Sin3A, the yeast HDAC1 homologue, Rpd3, and the Sin3A-associated protein, RbAp48 (RBBP4) (Figure 1.6Bii); the second possesses Sin3A-independent HDAC activity (Figure 1.6Biii) and the third complex lacks HDAC activity entirely (Figure 1.6Biv) [137]. The Sin3A-associated N-CoR/SMRT complex (Figure 1.6Bii) purifies as an eight-protein complex including: p110, p30, p100, p70, RbAp48, Rpd3, Sin3A and N-CoR/SMRT itself. Rpd3 and RbAp48 are more abundant than the other accessory proteins, in a ratio of 2:1 and 4:1, respectively, with N-CoR/SMRT, which may suggest these proteins function as the catalytic subunit and histone-interacting subunit, respectively. Complex two (Figure 1.6Biii), consists of four polypeptides, p115, p60, p52 and N-CoR/SMRT, with a stoichiometry of 1:1:2:4, respectively. No HDAC has been identified in the core complex, suggesting deacetylase activity may arise from an indirect association with an HDAC that escaped purification. Complex three (Figure 1.6Biv) also consists of four polypeptides: N-CoR/SMRT, p120, p65 and p29, with a stoichiometry of 2:3:1:1, respectively, predicting a mass of 1 MDa. The role of multiple N-CoR/SMRT subunits may indicate that the complexes must interact with multiple targeting proteins or additional co-repressor complexes *in vivo* [137].

Using mass spectrometry and Western blotting techniques, additional novel components of the N-CoR/SMRT complex have been identified, including the SWI/SNF-related proteins BRG1, BAF 170, BAF 155, BAF 47, and the co-

repressor KAP-1 that is involved in silencing heterochromatin [144] (Figure 1.6Bv). While this N-CoR/SMRT complex contains only HDAC3, another N-CoR/SMRT complex identified by the same group contained predominantly HDAC1 and HDAC2, as well as several other subunits that are found in the Sin3A/HDAC complex (Figure 1.6Bvi) [144]. While multiple N-CoR/SMRT complexes have been identified, additional biochemical studies are necessary to elucidate the full repertoire of biological functions and mechanisms.

The mechanism of N-CoR/SMRT repression is dependent on the N-terminus of the protein, which contains two SANT domains, while the majority of transcription factor binding occurs in the C-terminus of the protein [90]. N-CoR/SMRT function is not confined to T_3R and RAR; they confer repressor activity on many transcription factors, including NFκB and activator protein-1, which stimulates proliferation. Identification of an increasing number of binding partners suggests these repressors are involved in regulating a variety of cellular processes, including not only proliferation, but also development and apoptosis. Hormone binding regulates N-CoR/SMRT complexes and their association with nuclear receptors, but additional cell signalling pathways, including ERK and PKA-mediated signalling, can also culminate with N-CoR/SMRT regulation. Indeed, treatment of L-throxine to activate the ERK MAPK signalling pathway caused SMRT dissociation from the thyroid hormone receptor, TRβ1, in a hormone-independent manner [145]. Depending on the binding partner or signalling pathway initiated, the repressors can be degraded or relocalised to the cytoplasm to modulate access to transcription factors [90].

1.5.3 NuRD

NuRD is an ATP-dependent repressor protein. In addition to binding HDAC1 and HDAC2, Rb associated proteins, RBBP4 (RbAp48) and RBBP7 (RbAp46), the ATPases, Mi-2, either the α- or –β isoform, also form part of the core complex. These proteins catalyse ATP hydrolysis to initiate chromatin remodelling and deacetylase activity and therefore play a key role in gene regulation [131]. The NuRD repressor complex also involves the methyl-CpG-binding domain proteins, MBD2 and/or MBD3 (p32) and metastasis-associated proteins, MTA1–3 (Figure 1.6Ci) [135].

The MBD family of proteins mediate interactions with methylated CpG dinucleotides, except for MBD3 (p32), which contains two point mutations to prevent nucleotide binding. The methylation state of DNA is related to transcriptional activity, with nucleotides in silenced genes often possessing this post-translational modification [135]. MBD2 and MBD3 can dimerise but also mediate additional protein-protein interactions to further modulate gene targeting of the complex.

MTA2 is constitutively expressed and is therefore considered to mediate NuRD housekeeping gene regulation, whereas MTA3 and MTA1 (p70) are expressed in a tissue-specific manner, generating more specialised roles for the repressor complex [90]. The binding of accessory proteins further modulates NuRD function and interactions of transcription factors with particular core NuRD complex components is also responsible for tissue and cell-specific functions [131].

Mi-2α and Mi-2β have distinct functions, but they can also co-exist within the same NuRD complex [131]. As shown in Figure 1.6Cii, the binding of two Mi-2-containing complexes, as a dimer, may be dependent on the dissociation of MBD3 (p32). Furthermore, Mi-2 isoforms may also associate with either HDAC1 or HDAC2, which can subsequently dimerise [138, 146].

Purification of an endogenous HDAC2 complex from HeLa cell extracts by immunoaffinity chromatography identified a previously uncharacterised protein of 110 kDa. Sequence analysis by peptide mass spectrometry, identified significant homology of this protein to polyamine oxygenase. The potential association of the NuRD complex with polyamine-oxygenase-like protein, shown in Figure 1.6Ci by a reversible reaction, suggests a potential role for the NuRD complex in redox reactions [138].

1.5.4 Sin3

Mammalian Sin3 exists as three isoforms generated from two different genes, *SIN3A* and *SIN3B* and is considered to be a master scaffold, binding and coordinating a series of core proteins in order to repress transcription [139]. Sin3 comprises four highly conserved paired amphipathic helix (PAH) domains, which mediate protein-protein interactions and are essential for full repressor function, as well as an HDAC interaction domain (HID) and a highly conserved region (HCR) in the C-terminus, which is less well characterised [147]. Sin3 itself has no intrinsic DNA binding ability and therefore relies upon binding different transcription factors in order to regulate specific genes. The enzymatic activity of the complex arises from binding HDAC1 and HDAC2, which along with five other proteins comprise the

core mammalian Sin3 complex, which is shown in Figure 1.6D and Figure 1.7 [148]. Included in the core complex are SAP30, SAP18, and SDS3 as well as RBBP4 (RbAp48) and RBBP7 (RbAp46), which are also components of the NuRD complex. These repressors therefore have a similar core unit, to which the addition of repressor-specific proteins enables more specialised functions. Sin3 also recruits other repressor complexes, including SMRT, N-CoR and REST/Co-REST, which bind to PAH1 [149-151].

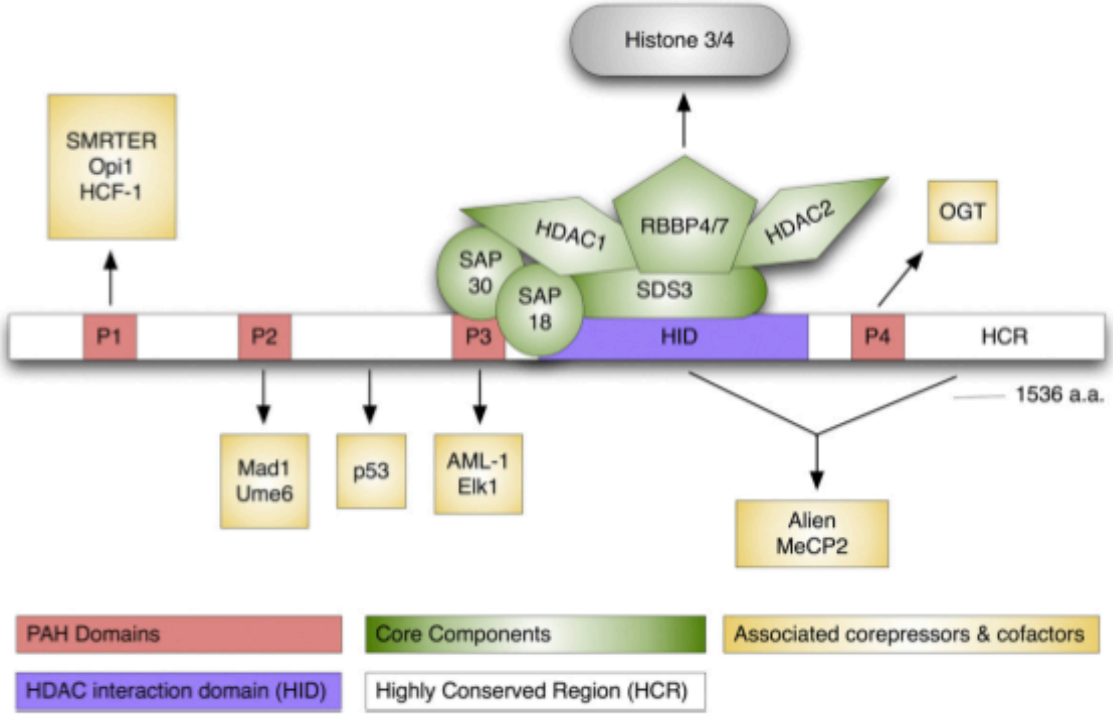


Figure 1.7 Sin3 structure and binding partners

Sin3A comprises six domains. The core repressor complex, shown in green, binds at the HID, with paired-amphipathic helix (PAH) domains mediating protein-protein interactions with associated co-repressors and co-factors. The highly conserved region (HCR) remains poorly characterised. The core Sin3 complex comprises HDAC1, HDAC2, RBB4 and 7, SDS3, SAP 18 and SAP30, which can also bind HDAC3 and HDAC4 as accessory proteins [139].

Providing a functional platform for class I HDAC activity means repressor complexes, like HDACs themselves, play important roles in physiological and pathophysiological processes by regulating gene expression. The recruitment of HDACs and transcription factors to form a multi-protein repressor complex allows for additional layers of transcriptional regulation. The interplay between transcriptional activation and repression of specific genes is crucial in developmental processes and is often disrupted in disease. During pathological cardiac hypertrophy, cardiac remodelling ensues following the repression of adult cardiac genes and the upregulation of foetal genes. Cancer is often associated with repression of pro-apoptotic proteins, as well as proliferation inhibitors, such as p21. The involvement of HDACs and their repressor complexes in these processes are therefore implied and the identification of GRK5 as an HDAC activator, suggests the kinase may also participate in HDAC regulation. Furthermore, the involvement of both HDAC repressor complexes and GRK5 in cardiac hypertrophy and cancer suggests their potential coordination in disease pathogenesis.

1.6 Aims of my thesis

In light of the discovery of the NLSs and NESs of GRK5, numerous nuclear substrates of the kinase have been identified. HDAC5 was the first nuclear substrate to be discovered, whose GRK5-mediated phosphorylation acts in a pro-hypertrophic manner and results in the upregulation of MEF2 transcriptional activity. Given the role of HDACs in gene repression, I aimed to investigate whether GRK5 could regulate transcription in a direct manner. Many diseases are associated with aberrant gene expression and GRK5 expression levels vary in disease states. We

therefore hypothesised that GRK5 regulates transcription and could thereby influence disease pathogenesis.

In this thesis I identify class I HDACs and the transcriptional repressor protein, Sin3A, as novel binding partners of GRK5. I explain attempts to map the binding sites of HDAC1 and Sin3A on GRK5 and explore the potential role of GRK5 as a transcriptional regulator in the context of colon cancer. I elucidate a novel GRK5-dependent mechanism of Bcl-2 transcriptional repression in the colon cancer cell line, HT-29, which increases sensitivity to apoptosis-promoting chemotherapeutic agents.

Chapter 2. Materials and methods

Tissue culture dishes were obtained from Nunc, culture medium from Gibco BRL, plastic-ware from Falcon or Sterilin and other reagents from Sigma, unless otherwise stated. Buffer ingredients, antibodies, cDNAs, small inhibiting RNAs (siRNAs) and micro RNAs (miRNAs) referred to in this report are listed in Tables 2.1–2.4.

2.1 Cell culture

All cell lines (HeLa, HEp2, HT-29, SW620, OVCAR3, IGROV-1, A2780 and PC3) were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% calf serum and penicillin (100 IU/ml)/ streptomycin (100 µg/ml). Cells were maintained in a humidified incubator at 37°C, 5% CO_2. Confluent monolayers were passaged every 3–4 days by trypsinising and replating at a ratio of 1:10. **Cell freezing and thawing:** To freeze cell stocks, two 70% confluent 9 cm plates were trypsinised and resuspended per 1 ml of freezing medium (DMEM, 20% foetal calf serum, 10% dimethylsulphoxide (DMSO) and penicillin (100 IU/ml)/ streptomycin (100 µg/ml)). After 1 week at -80°C, cells were transferred to liquid nitrogen. To recover stocks, aliquots were rapidly thawed at 37°C and plated onto a 9 cm plate in 15 ml culture medium.

2.2 Transfection of cDNA, siRNA and miRNAs

Cells were transiently transfected using Fugene HD (Roche). cDNAs, siRNAs or miRNAs were added to 100 µl of serum-free DMEM along with 3 µl of Fugene per 1

µg DNA. Samples were mixed by gentle agitation and incubated at room temperature for 20 minutes. The media/cDNA/Fugene mixes were then added drop-wise to 50% confluent cells and unless otherwise stated, 24 hours was allowed for protein expression.

2.3 Preparation of cell lysates and nuclear extracts

Cells in 9 cm dishes were washed twice with cold phosphate buffered saline (PBS) on ice and lysed by addition of 1 ml of the appropriate chilled lysis buffer. A cell scraper was used to remove cell material from the dishes and lysates were transferred to a 1.5 ml tube. After 15 seconds sonication (Branson Sonifier 450, setting 6), lysates were cleared by centrifugation at 14,000 rpm (Heltich bench top centrifuge Mikro 20) for 5 minutes. Protein concentrations were determined using the BioRad protein assay according to manufacturer's instructions and lysates were stored at -20°C. **For preparation of nuclear extracts:** cells were harvested in 1 ml cold nuclear lysis buffer, vortexed for 10 seconds and incubated on ice for 15 minutes. Nuclei were isolated by spinning the lysates through 4 ml cold sucrose cushion at 1300 g for 10 minutes at 4°C (Sorvall TC-6 centrifuge). The nuclei pellet was then resuspended in 1 ml cold Tris-HCL, pH7.5, containing 10 mM NaCl and clarified by centrifugation at 1300 g for 10 minutes at 4°C (Sorvall TC-6 centrifuge). Nuclei were extracted by suspension in 100–200 µl of extraction buffer and sonicated for 30 seconds before incubation on ice for 30 minutes. Samples were centrifuged at 10,000 g for 10 minutes at 4°C (Sorvall TC-6 centrifuge) and the supernatants containing crude nuclear extracts stored at -80°C until use.

2.4 Co-Immunoprecipitation

Volumes of cell lysates harvested in GTPase buffer, containing 200 µg protein, were incubated with the appropriate antibody for 1 hour rotating at 4°C. Protein A/G sepharose beads (Santa Cruz) were washed in GTPase lysis buffer and 30 µl of a 50% suspension was added to each sample before incubation for a further hour, rotating at 4°C. The samples were then spun down at 200 g (1500 rpm Heltich bench top centrifuge Mikro 20) for 2 minutes, the supernatant aspirated and 1 ml GTPase lysis buffer added to wash the beads. This wash cycle was repeated 3 times before adding 25 µl sodium dodecyl sulphate (SDS) reducing buffer to each sample in preparation for analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

2.5 Immunofluorescent labeling

Coverslips with cell monolayers were fixed in 4% paraformaldehyde (TAAB)/ PBS for 20 minutes before quenching for 10 minutes in 0.27% NH_4Cl/ 0.37% glycine in PBS. 1% bovine serum albumin (BSA) (First Link UK Ltd.)/ 0.2% Saponin/ PBS was then added for 30 minutes to block and permeabilise fixed cells. Primary antibody incubations were performed for 1 hour at room temperature in 1% BSA/ 0.2% Saponin. Cells were subsequently washed 3 times with 1% BSA/ 0.2% Saponin before incubation with the appropriate secondary antibody for 30 minutes at room temperature. Following 3 more washes in 1% BSA/ 0.2% Saponin, cells were washed twice in PBS and coverslips were mounted on slides in 90% glycerol/ PBS/ 3% N-propyl-galate. Confocal images were taken at room temperature with Leica ACS Apo 40x and 63x oil immersion lenses. A Leica TCS SPE confocal

microscope and LAS AF software was used to acquire the images. Images were optimised for contrast in Adobe Photoshop but no further manipulations were made. **For HEp2 cells:** 24–48 hours post-transfection, cells were treated or not with 25 μM Ca^{2+} ionophore, A23187 (Calbiochem) for 15 minutes in medium supplemented with 2 mM $CaCl_2$ at 37°C, 5% CO_2. Cells were stained as detailed above.

2.6 Direct binding assay

10 μl purified glutathione S-transferase (GST) or GST-HDAC1, GST-HDAC3 or GST-HDAC8 was immobilised on glutathione sepharose beads (GE Healthcare) in 200 μl protein binding buffer. 10 μl of GRK5 purified from SF9 cells was added and samples incubated, rotating, for 1 hour at 4°C. Beads were then washed 4 times in protein binding buffer before adding 25 μl SDS reducing buffer to each sample ready for analysis by SDS-PAGE. GRK5 binding was detected using an anti-GRK4-6 antibody. **For GST-Sin3A deletion mutant pull downs (Figure 3.6) and for screening GRK5 mutants for direct binding to HDAC1 and Sin3A (Figure 4.7 and 4.8):** Wildtype or mutant GRK5 (M1: E355A/V356A, M2: D485A, M3: D486A, M4: D487A, M5: D485/486/487A or GRK5ΔNLS) was *in vitro* translated and radiolabelled with [^{35}S]-methionine using the Promega quick-coupled transcription/ translation system according to manufacturer's instructions. 10 μl *in vitro* translated wildtype GRK5 was incubated with GST or GST-Sin3A deletion mutants for Sin3A pull downs (Figure 3.6). For the GRK5 mutant pull-downs, 10 μl *in vitro* translated wildtype or mutant GRK5 was incubated with GST, GST-HDAC1 or GST-Sin3A-545-1157 (Figure 4.7 and 4.8). The incubations and subsequent washing steps were carried out in 200 μl protein binding buffer. SDS-PAGE gels were incubated with Autofluor (National Diagnostics), the Autoradiographic Image Intensifier, as per

instructions, for an hour, and then placed on 2 sheets of filter paper, covered with Saran wrap and dried using a Model 583 Gel Dryer (BioRad) at 80°C for 90 minutes before being exposed to film (Biomax ML, Kodak) at -70°C overnight. Film was developed using an Agfa automatic film processor. **For direct binding of HDAC1 and HDAC2 to GRK5 (Figure 3.4):** 10 µl *in vitro* translated Flag-HDAC1 or Flag-HDAC2 was incubated with GST or GST-GRK5 in 200 µl NETN buffer. Subsequent washing steps were carried out in H Buffer. SDS-PAGE gels were dried down and analysed by autoradiography.

2.7 Peptide array overlays

The GRK5 peptide arrays were kindly received from Dr George Baillie (Glasgow University). GRK5 peptide libraries of overlapping 25-mer peptides each shifted along by five amino acids were produced by automatic SPOT™ synthesis onto Whatman 50 cellulose membranes using 9-fluorenylmethyloxycarbonyl chemistry with the AutoSpot-Robot ASS 222 (Intavis Bioanalytical Instruments) [119, 120]. The arrays were activated by immersion in 100% ethanol, rinsed in Tween/Tris-buffered saline (TTBS) and incubated in blocking buffer for one hour at room temperature. The interaction of spotted peptides with target proteins was investigated by overlaying the membranes with 10 µg/ml recombinant fusion protein in 1–5% blocking buffer overnight at 4°C. Detection of bound fusion proteins is as per Western blotting, using an anti-GST primary antibody and the relevant secondary antibody. **For alanine scanning substitution arrays:** Alanine scanning substitution analyses were performed to determine specific amino acid residue(s) important for mediating the binding event. Amino acid residues from selected peptide sequences were replaced with alanine one at a time unless the indicated

amino acid was alanine, when it would be substituted for aspartate. Control spots (*) consisting of full length, unmutated peptides known to bind to the protein of interest were also included for comparative purposes. The arrays were immunolabeled and detected by autoradiography, as described above.

2.8 Gal4 luciferase reporter assay

Gal4 transcriptional activity was recorded using the Dual-Luciferase® Reporter Assay system (Promega), according to the manufacturer's instructions. Briefly, the minimal promoter-luciferase reporter containing a GAL4 DNA-binding domain (DBD) alone (GAL4-DBD), or fused to a GRK5 construct (Gal4-DBD-GRK5), which were kindly received from Dr Mark Scott, were transfected into HT-29 cells in 6 well plates. The cells were additionally co-transfected with pFR-luc, a firefly luciferase expression plasmid in the cytomegalovirus (CMV) vector under the control of the Gal4-DBD, and pRL-CMV, a constitutively expressed *Renilla* luciferase CMV plasmid, as a measure of transfection efficiency. Luciferase activities were determined 24 hours post transfection according to manufacturer's instructions. The cells were harvested on ice in 250 µl passive lysis buffer (Promega) and 20 µl cell lysate incubated with 100 µl luciferase assay substrate and firefly luminescence recorded for 15 seconds using a Turner TD-20e luminometer. 100 µl Stop and Glo^{TM} reagent was added and the specific luminescence from *Renilla* luciferase was recorded for an additional 10 seconds. The experiment was repeated as a triplicate and the average firefly luminescence calculated relative to that of *Renilla*.

2.9 PCR

2.9.1 Site directed mutagenesis

The QuikChange® site-directed mutagenesis kit (Stratagene) was used according to manufacturer's instructions to make point mutants in the potential HDAC1 and HDAC1/Sin3A binding sites on GRK5. A bovine GRK5 construct (pRK5-GRK5) was used as template to create the mutants M1–5, as outlined in Table 4.2. GRK5 sequencing primers, to enable sequencing of the entire GRK5 cDNA following PCR/mutagenesis are as follows:

GRK5seqF1 400 – CTTCAGAAACCCTGCAAAGA – 419

GRK5seqF2 814 – AAGTTCCACATTTACAACAT – 833

GRK5seqF3 1201 – GTGTTGGAGACAGAGGAGGT – 1220

GRK5seqF4 1556 – AGTGCTTTAAGGAGCTGAAC – 1575

To create the potential GRK5ΔHDAC1 mutant (M1), amino acids 55 and 56 were mutated to alanine residues using the following primers; sense primer 5'–C GTT GGC TAC ATG GCT CCA G**C**G G**C**C CTG AAC AAC CAG AGG TAT GG–3', anti-sense primer 5'–CC ATA CCT CTG GTT GTT CAG G**G**C C**G**C TGG AGC CAT GTA GCC AAC G–3'. To create the potential GRK5ΔHDAC1ΔSin3A mutants, amino acids 485 (M2), 486 (M3), 487 (M4) or 485–487 (M5) were mutated to alanine residues to produce mutants M2–M4 using the following primers; M2: sense primer 5'–C CTG GAC CAC ACG G**C**C GAC GAC TTC TAC TCC–3', anti-sense primer 5'–GGA GTA GAA GTC GTC G**G**C CGT GTG GTC CAG G–3'; M3: sense primer 5'–C CTG GAC CAC ACG GAC G**C**C GAC TTC TAC TCC–3', anti-sense primer 5'–GGA GTA GAA GTC G**G**C GTC CGT GTG GTC CAG G–3'; M4:

sense primer 5'–GAC CAC ACG GAC GAC G**C**C TTC TAC TCC AAG TTC TCC–3', anti-sense primer 5'–GGA GAA CTT GGA GTA GAA G**G**C GTC GTC CGT GTG GTC–3'; M5: sense primer 5'–GGC GTC AAC CTG GAC CAC ACG G**C**C G**C**C G**C**C TTC TAC TCC AAG TTC TCC–3', anti-sense primer 5'–GGA GAA CTT GGA GTA GAA G**G**C G**G**C G**G**C CGT GTG GTC CAG GTT GAC GCC–3'. Nucleotides in bold encode the mutated amino acids.

PCR was carried out as follows:

2 µl Template (10 ng)

5 µl 10X Reaction buffer

1.25 µl Sense primer (100 ng/µl)

1.25 µl Anti-sense primer (100 ng/µl)

1 µl dNTPs (10 mM)

1 µl Pfu polymerase

38.5 dH_20

_____

50 µl

PCRs were cycled in a PTC-2000 Peltier Thermal Cycler (MJ Research) as followed:

1. 95°C, 30 seconds
2. 95°, 30 seconds
3. 55°C, 60 seconds
4. 68°C, 11 minutes
5. Return to step 2 for 17 more cycles then step 6.

6. 4°C forever.

Following PCR, 1 µl DpnI was added to the reaction and incubated at 37°C for 1 hour to cleave methylated parent DNA. 5 µl of each PCR reaction was then transformed into competent *Escherichia coli (E. coli)* as detailed in section 2.11.4.

2.9.2 Quantitative RT-PCR (qRT-PCR)

HT-29 cells were transfected with 10 µg GRK5 siRNA or scrambled control for 72 hours. RNA was then extracted from cells using the QIAGEN RNAeasy kit with cells homogenised using the QIAshredder kit (QIAGEN), according to manufacturer's instructions. Prepared RNA samples were reverse-transcribed into cDNA using SuperScript® Reverse Transcriptase II (Invitrogen) according to the manufacturer's instructions. 6.25 µl cDNA samples at 150 ng/µl were then amplified by qPCR using the DyNAmo flash SYBER green QPCR kit (Thermo Scientific). 12.5 µl SYBER green was added to the cDNA along with 6.25 µl primer pairs (1.2 µM). mRNA levels of GRK5, Bcl-2 and actin were analysed using appropriate forward and reverse primer pairs (IDT Technologies). GRK5: forward primer 5'–GAC CAC ACA GAC GAC GAC TCC–3', reverse primer 5'–CGT TCA GCT CCT TAA AGC ATT–3'; Bcl-2: forward primer 5'–GCG ACT CCT GAT TCA TTG–3', reverse primer 5'–AGG TGC GTT TCC CTG TA–3'; actin: forward primer 5'– TTG TGG TGA AGC TGT AGC C–3', reverse primer 5'–GCG AGA AGA TGA CCC AGA T–3'. PCR reactions were performed using the Eppendorf Mastercycler® ep realplex^2 with 40 cycles of the following condition:

95°C for 10 seconds

55°C for 15 seconds

72°C for 15 seconds

Results were analysed using the Realplex software.

2.10 Fluorescence-activated cell sorting (FACS)

HT-29 cells were split into 12-well plates and transfected and treated 24 hours later, as indicated in Figure 5.8. Cells were harvested by trypsinisation and recovered in 500 µl DMEM. Cells were spun down at 1000 rpm (Heltich bench top centrifuge Mikro 20) for 5 minutes and the cell pellets resuspended in 500 µl DMEM containing 0.25 µg 7-aminoactinomycin D Viability Staining Solution (7-AAD, e-Bioscience). 7-AAD is a fluorescent DNA intercalator that is selectively excluded from live cells and is therefore used as an indicator of cell death. Samples were analysed within 30 minutes of incubation using a BD LSR II cytometer. The percentage of cells positively stained with 7-AAD was recorded, with 10,000 cells analysed per sample. Unstained cells were used as negative controls.

2.11 Other techniques

2.11.1 SDS-PAGE

SDS-PAGE was performed using the Hoefer Scientific Instruments vertical slab gel unit SE 600 gel system. 10% separating gel was cast between two glass plates by polymerising 30% (v/v) acrylamide in 0.375 M Tris-HCl pH8.8 and 1% SDS, using 0.3% (v/v) ammonium persulphate and 0.07% (v/v) N, N, N', N'-tetramethyl-ethylenediamine (TEMED). A 4% stacking gel was cast above the separating gel

by polymerising 4% (v/v) acrylamide in 0.12 M Tris-HCl pH6.8 and 1% SDS, using 0.1% (v/v) ammonium persulphate and 0.1% (v/v) TEMED. Samples were prepared by addition of 25 µl SDS reducing buffer to 10 µg of lysate or immunoprecipitate and heated at 60°C for 10 minutes. Samples were loaded onto the gels and the gels were subjected to a constant voltage of 300 V for 1 hour in gel running buffer. For direct binding experiments, radioactive gels were dried onto 3mm Whatman paper overnight and exposed to film at -80°C for 48 hours.

2.11.2 Western blotting and immunodetection

Proteins separated on SDS-PAGE gels were transferred onto nitrocellulose membrane (Hybond-ECL, Amersham) using a semi-dry electrophoretic transfer unit (V20-SDB). The gel and membrane were soaked in transfer buffer, placed between 6 soaked pieces of filter paper and subjected to a constant current of 0.8 mA per cm^2 membrane for 1 hour 45 minutes. Membranes were then incubated in blocking buffer for 1 hour at room temperature followed by incubation for 1 hour with the appropriate primary antibody diluted in blocking buffer, or overnight at 4°C. Following 5 washes over a 30 minute period in TTBS, a horseradish peroxidase-conjugated secondary antibody (Amersham), diluted in TTBS, was added to the immunoblots for 1 hour. The blots were then washed as before. Sufficient ECL reagent (Amersham) was added to cover the membrane and incubated for 1 min. Bound antibody was detected by exposing the immunoblot to film (Biomax ML, Kodak) for the required time and the film was developed using an Agfa automatic film processor and quantified using a BioRad densitometer.

2.11.3 Coomassie staining of SDS-PAGE gels

To stain proteins, the SDS-PAGE gel was covered in Coomassie stain, heated for 1 minute at 750W in a microwave and allowed to cool at room temperature on a shaker. The gel was then de-stained using Coomassie de-stain until proteins were visible and identifiable by comparison to molecular weight markers.

2.11.4 Bacterial transformation and plasmid DNA purification

TOP10 (Invitrogen) chemically competent *E. coli* were thawed on ice and mixed by hand. 20 µl of cells were aliquoted into a pre-chilled 1.5 ml Eppendorf tube containing 50 ng of plasmid cDNA and incubated on ice for 30 minutes. The bacteria were then heat shocked at 37°C for 5 minutes before incubation on ice for 2 minutes. 1 ml pre-warmed super-optimal broth with catabolite repression (SOC) medium (Invitrogen) was added and the bacteria were incubated at 37°C for 1 hour with shaking at 200 rpm (Kuhner ISF-1-W bacterial incubator). The bacteria were then plated onto Luria Broth (LB) agar plates containing 100 µg/ml ampicillin or 25 µg/ml kanamycin, as appropriate, and incubated at 37°C overnight. The next day, single colonies were picked from the plate and grown overnight at 37°C, shaking at 200 rpm (Kuhner ISF-1-W bacterial incubator), in 500 ml of LB medium containing 100 µg/ml ampicillin or 25 µg/ml kanamycin as appropriate. The following day, the bacteria were pelleted and the plasmid DNA was extracted using a QIAprep Spin Maxiprep kit (QIAGEN). The concentration of the plasmid DNA was determined by measuring A_{260} using an Ultraspec 2000 spectrophotometer (Pharmacia Biotech).

2.11.5 Purification of GRK5

GRK5 was overexpressed in baculovirus-infected SF9 cells by Dr Julie Pitcher. Cell pellets were thawed, supplemented with fresh protease inhibitors (40 µg/ml phenylmethanesulfonyl fluoride (PMSF), 1 mM benzamidine), and homogenised with 10 strokes of a tightglass Dounce homogeniser on ice. All subsequent manipulations were performed at 4°C and all buffers contained protease inhibitors as above. The homogenate was spun at 43,000 g for 20 minutes and the resulting pellet was re-homogenised with 50 ml of purification buffer with 20 mM NaCl and spun as before. The two supernatants were pooled and passed through a 10 ml column of S-Sepharose (Pharmacia) at a flow rate of 1 ml/min. Most proteins failed to bind to the resin. The column was washed with 50 ml of buffer A with 20 mM NaCl and eluted with a linear 100 ml gradient of 20–750 mM NaCl in purification buffer. Fractions were assessed for GRK5 and contamination by Coomassie staining of 10% SDS-PAGE gels and those containing GRK5 and the fewest protein contaminants were pooled (20 ml), diluted with buffer A to below 100 mM NaCl, and applied to a 10 ml column of heparin-Sepharose (Pharmacia) at 1 ml/min. The column was washed with 50 ml of purification buffer with 150 mM NaCl and eluted with a linear 100 ml gradient of 15–1500 mM NaCl in purification buffer. Fractions containing purified GRK5, as assessed by SDS-PAGE Coomassie staining, were pooled and concentrated to 1 ml in a Centriprep 30 spin concentrator (Amicon). Purified GRK5 was stored at 4°C or at -20°C in 50% glycerol. Final purity of purified GRK5 was assessed by Coomassie staining.

2.11.6 GST-GRK5, GST-HDAC and GST-Sin3A deletion mutant fusion protein purification

BL21 (Invitrogen) *E. coli* were transformed as described for TOP10. 100 ml starter cultures were diluted 1/10 in room temperature LB and left at 37°C shaking for an hour before being moved to 20°C for 1 hour, shaking at 200 rpm. Isopropyl-β-D-thio-galactoside (IPTG) was then added to the final concentration of 0.03 mM for 18 hours to induce fusion protein expression. After pelleting, the bacteria were resuspended in 25 ml PBS containing the protease inhibitors 40 µg/ml PMSF and 1mM benzamidine. Lysates were sonicated twice for 2 minutes, on ice. 1% Triton X-100 was then added to the lysates before being cleared by centrifugation for 30 minutes at 39,500 g, at 4°C. 1 ml of 50% glutathione sepharose beads (GE healthcare) was added to the supernatants and incubated for 2 hours at 4°C on a rotating wheel. The beads were then washed 3 times with cold 50 mM Tris pH8.0, 1% Tween, 1% Triton X-100 and once with cold 50 mM Tris pH8.0. For the peptide arrays, GST-fusion proteins were eluted from the glutathione sepharose beads with 25 mM glutathione in 50 mM Tris pH8.0 containing protease inhibitors and dialysed overnight in TTBS. Samples were run on a Coomassie gel with BSA standards to determine fusion protein purity and concentration.

2.11.7 In vitro translation

For direct binding experiments, proteins were *in vitro* translated and radiolabelled with [^{35}S]-methionine using the Promega quick-coupled transcription/translation system according to manufacturer's instructions. The RNA polymerase, nucleotides, salts and Recombinant RNasin® Ribonuclease Inhibitor were combined with rabbit

reticulocyte lysate to form a single TnT® Quick Master Mix. The TNT® Quick Coupled Transcription/Translation System is available in two configurations for transcription and translation of genes cloned downstream from either the T7 or SP6 RNA polymerase promoters. The SP6 or T7 TnT® Quick Master Mix was rapidly thawed after removing from storage at -70ºC and placed on ice. 0.2–2.0 µg cDNA was incubated with 40 µl TnT® Quick Master Mix along with 2 µl [^{35}S]-methionine (1,000 Ci/mmol at 10 mCi/ml) for 30–60 minutes. The *in vitro* translated proteins were then stored at -80°C until use.

2.12 Statistical analysis

Results were analyzed using the student's two-sample T-test to determine whether measurements made on two populations were different from each other. The null hypothesis proposed that the two populations were the same in every case.

Table 2.1 Primary antibodies

Antigen	Source	Product number	Dilution for Western	Volume for IP (µl)	Dilution for Immunofluorescence
β-Actin	Mouse	Santa Cruz SC-81178			
Bcl-2	Mouse	Santa Cruz SC-130308	1:1000		
Active cleaved caspase 3	Rabbit	Cell Signaling Technology 9661S			1:100
Cleaved PARP-1	Rabbit	Santa Cruz SC-23461	1:1000		
ERK	Rabbit	Sigma M5670	1:20,000		
Flag	Mouse	Sigma F3165	1:1000	3	
GRK2	Rabbit	Santa Cruz SC-562	1:500		
GRK4-6	Mouse	Upstate 05-466	1:1000		1:300
GRK5	Rabbit	Santa Cruz SC-565	1:1000		1:50
GST	Rabbit	Sigma Aldrich G7781	1:8000		
HA	Rat	Roche 1867423	1:1000		
HDAC1	Mouse	Santa Cruz SC-81598	1:1000		
HDAC2	Mouse	Santa Cruz	1:1000		

		SC-81599			
RBBP4	Rabbit	Abcam ab1765	1:1000		
Sin3A	Mouse	Santa Cruz SC-5299	1:1000	5	
Myc	Mouse	Millipore 05-724	1:1000		1:100

Anti-mouse and anti-rabbit IgG HRP-conjugated secondary antibodies were from Sigma (sourced from sheep, 1:20,000). For immunofluorescence, anti-mouse and anti-rabbit IgG Alexa Fluor® 488 and 594 conjugated secondary antibodies were from Molecular Probes (sourced from donkey, 1:700).

Table 2.2 Buffers

Buffer Name	Ingredients
Blocking buffer	5% skimmed milk powder made up in TTBS
Buffer A	34% sucrose w/w 65 mM NaCl 2 mM $MgCl_2$ 10 mM Tris-acetate buffer, pH 7.4 0.1 mM EDTA 500 µM PMSF 1 µM Leupeptin 1 µM Pepstatin
Coomassie stain	40% MeOH 10% acetic acid 0.05% Brilliant Blue G
Coomassie de-stain	40% MeOH 10% acetic acid
Extraction buffer	50 mM HEPES pH 7.5 420 mM NaCl 0.5 mM EDTA 0.1 mM EGTA 10% glycerol
Gel running buffer	2 M glycine 0.25 M Tris-HCl 0.03 M SDS
GTPase lysis buffer	10% glycerol 50 mM Tris-HCl pH8 150 mM NaCl 1% TX-100 2 mM EDTA 40 µg/ml PMSF
H buffer	20 mM HEPES pH7.7 50 mM KCl

	20% glycerol 0.1% NP-40
NETN buffer	20 mM Tris pH8.0 100 mM NaCl 1 mM EDTA 0.5% NP-40
Nuclear lysis buffer	10 mM Tris pH7.5 10 mM NaCl 15 mM MgCl2 250 mM sucrose 0.5% NP-40 0.1 mM EGTA
PBS	137 mM NaCl 2.7 mM KCl 4.3 mM Na_2HPO_4 1.47 mM KH_2PO_4 Adjust to a final pH of 7.4.
Protein binding buffer	20 mM HEPES pH7.8 1 mM MgCl2 10 µM ZnCl2 2 mM DTT 10% glycerol 0.05% Triton X-100 100 mM KCl 40 µg/µl BSA
Purification buffer	20 mM HEPES 2 mM EDTA 0.02% Triton X-100 pH7.2
SDS reducing buffer	25 mM Tris-HCl pH6.5 10% glycerol 8% SDS 5% β-mercaptoethanol

	A few grains of Brilliant Blue G
Sucrose cushion	30% sucrose 10 mM Tris pH7.5 10 mM NaCl 3 mM MgCl2
Transfer buffer	0.05 M Tris-HCl 0.04 M glycine 0.01 M SDS 20% MeOH
TTBS	50 mM NaCl 20 mM Tris-HCl pH8.0 0.1% Tween-20

Table 2.3 cDNA constructs

Construct Name	cDNA	Supplier	Reference
	GRKs		
GRK5	GRK5-pRK5	Dr Robert Lefkowitz	[152]
GRK5 M1 GRK5 M2 GRK5 M3 GRK5 M4 GRK5 M5	GRK5-pRK5	Miss K Lester	
GST-GRK5	pGEX-GRK5	Prof J Benovic	[57]
Gal4-DBD-GRK5	pFA-CMV-GAL4-DBD	Dr M Scott	[153]
	HDACs		
Flag-HDAC1 Flag-HDAC2 Flag-HDAC3	pcDNA3.1-Flag-hHDAC	Dr Stephane Emiliani	[154]
Flag-HDAC8	pCEP4F-Flag-HDAC8	Prof. Antonella Riccio	[155]
HA-HDAC4 HA-HDAC5 HA-HDAC6 HA-HDAC7 HA-HDAC9 HA-HDAC10	pcDNA-HA3.1 -HA-HDAC	Dr Saadi Khochbin	[156-158]
GST-HDAC1 GST-HDAC2 GST-HDAC3 GST-HDAC8	pGEX-KG-HDAC	Prof. Antonella Riccio	[159, 160]
	Sin3A		
Sin3A-N205 Sin3A-N479	pCS2+MT-mSin3A	Dr R Eisenman	[161]

Sin3A-N680 Sin3A-N1015			
GST-Sin3A 57-215 GST-Sin3A 215-404 GST-Sin3A 404-545 GST-Sin3A 545-1157	pGEX-KG-mSin3A	Dr. Martin Privalsky	[162]
Other			
GST	pGEX6p1-EV	Dr. S Nurrish	[163]
Gal4	pFA-CMV-GAL4-DBD	Dr M Scott	
Renilla luciferase	pRL.CMV	Dr M Scott	
Firefly luciferase	pFR-luc	Dr M Scott	

Table 2.4 siRNA and miRNA constructs

Construct name	Sequence	Supplier
GRK5 siRNA	5'–AAG CCG UGC AAA GAA CUC UUU–3'	Thermo Scientific
miR-135a-5p *mir*Vana® miRNA mimic	5'–UAUGGCUUUUUAUUCCUAUGUGA–3'	Invitrogen
miR-135a- 5p *mir*Vana® miRNA inhibitor	5'–UAUGGCUUUUUAUUCCUAUGUGA–3'	Invitrogen

Chapter 3. Class I HDACs and Sin3A are novel binding partners of GRK5

3.1 GRK5 interacts directly with class I HDACs

The GRKs are known canonically for their role in phosphorylating GPCRs, preventing coupling to heterotrimeric G proteins and triggering receptor desensitisation. As discussed in detail in chapter 1, GRKs are becoming increasingly recognised for their role in mediating G protein-independent signalling. The discovery that GRK5, and the other GRK4 subfamily members, contain a functional NLS and NES prompted research into a role for the kinase in the nucleus. The first nuclear substrate of GRK5 to be identified was HDAC5, whose phosphorylation stimulates its nuclear export [81]. HDACs regulate gene expression by catalysing the deacetylation of lysine residues on histones and work in concert with repressor complexes to target their enzymatic activity to specific genes. The phosphorylation of HDAC5 by GRK5 relieves the inhibition of the pro-hypertrophic transcription factor, MEF2, thus resulting in the upregulation of MEF2 target genes and the progression towards pathological cardiac hypertrophy [81].

The study of HDACs in the heart is largely concerned with their role as regulators of cardiac hypertrophy. Treatment with a pan-HDAC catalytic inhibitor, TSA, suppresses *in vitro* and *in vivo* models of cardiac hypertrophy [125, 126]. Despite targeting both classes of HDAC, the effect of TSA on cardiac hypertrophy is considered to be due to its action on class I HDACs, which are pro-hypertrophic and dominate over the anti-hypertrophic action mediated by class II HDACs [128,

164]. Indeed, treatment with the class I specific HDAC inhibitor, SK-7041, significantly reduces hypertrophic development in mice [129].

GRK5 is upregulated in patients with left ventricular overload disease, which is associated with HCM [87]. Moreover, the cardiac specific overexpression of GRK5 in mice potentiates pressure-overload induced hypertrophy and GRK5 knockout mice show delayed hypertrophy and preserved cardiac function. GRK5 is therefore playing a causative role in disease progression possibly via its role as an HDAC5 kinase [81, 165]. The NLS of GRK5 contains a functional DNA binding site, thus suggesting the kinase could also be playing a more direct role in controlling transcription [11]. In light of the causative roles played by both GRK5 and class I HDACs in hypertrophy, I wanted to investigate whether GRK5 could also contribute to disease progression in a class I HDAC-dependent fashion.

To test this hypothesis I performed co-immunoprecipitation experiments in HeLa cells, as this cell line is easy to maintain and transfect. I over expressed Flag-tagged class I HDACs or HA-tagged class II HDACs in HeLa cells with or without GRK5 and used an antibody against the epitope tag to immunoprecipitate the HDACs. GRK5 binding was detected by Western blotting using an anti-GRK5 antibody. Figure 3.1 clearly shows that GRK5 binds to class I HDACs, HDAC1, HDAC3 and HDAC8 (Figure 3.1A) relative to HDAC2 and all class II HDACs (Figure 3.1B).

GRK5 is an HDAC5 kinase [81], but as compared to class I HDACs, the interaction between GRK5 and HDAC5 is relatively weak (Figure 3.1B). Relationships between substrates and kinases are often transient, with dissociation occurring promptly after the phosphorylation event, thus ensuring the kinase is able to target multiple substrates. Such interactions can therefore be difficult to capture, particularly by co-immunoprecipitation. The poor interaction of GRK5 with class II HDACs, as shown in Figure 3.1, is therefore typical of a substrate/kinase relationship [166], thus suggesting that the GRK5-mediated regulation of this class may not extend beyond phosphorylation.

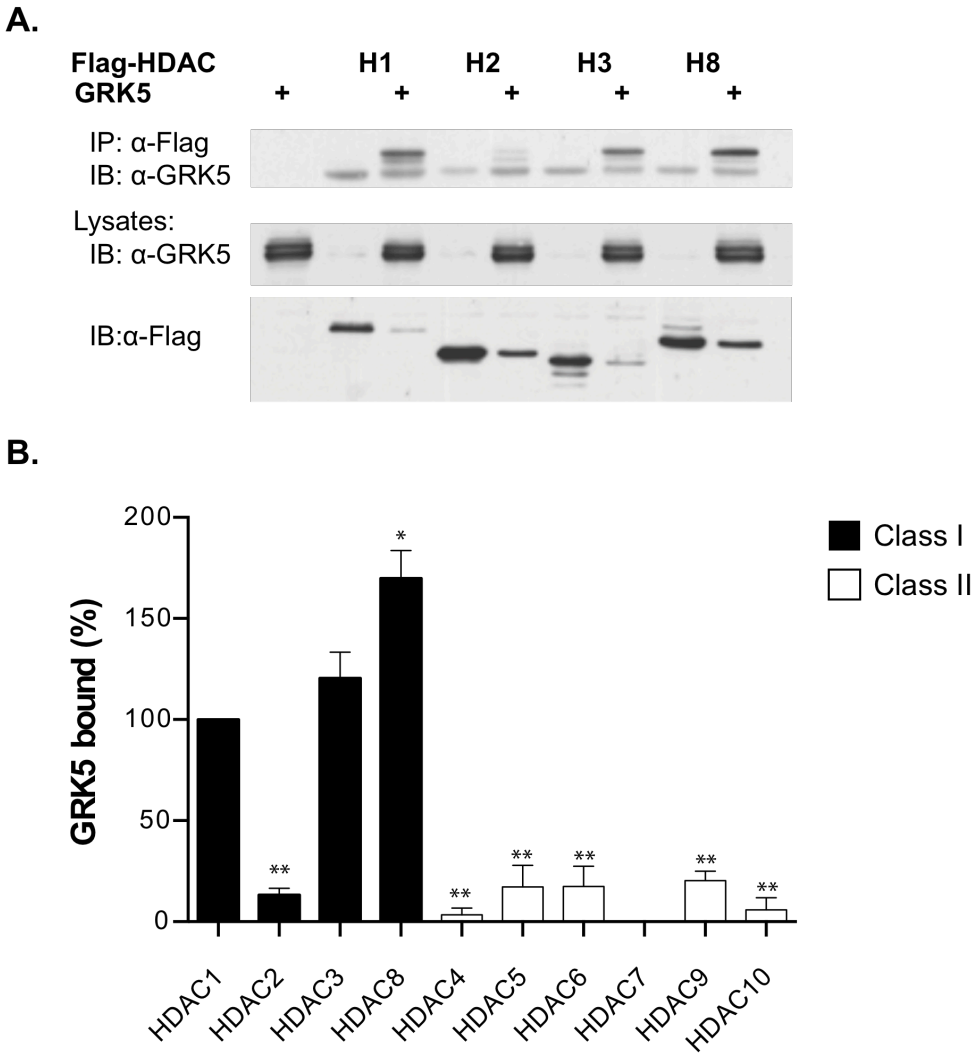


Figure 3.1 GRK5 binds class I HDACs

A. Representative Western blot showing the co-immunoprecipitation of Flag-tagged class I HDACs with GRK5. HeLa cells were transfected as indicated and protein expression confirmed by Western blotting (lysates) using an anti-Flag or anti-GRK5 antibody to detect HDACs or GRK5 respectively. Flag-tagged class I HDACs were immunoprecipitated (IP) and the amount of GRK5 bound to immunoprecipitated Flag-HDAC was detected by immunoblotting (IB). The blots shown are representatives of 3 separate experiments. **B.** Data quantified from co-immunoprecipitations involving class I and class II HDACs. The percentage of GRK5 co-immunoprecipitated per unit HDAC immunoprecipitated is normalised to the amount of co-immunoprecipitation seen for HDAC1. Error bars represent standard error of the mean from three separate experiments, with *P<0.001; **P<0.05 relative to the amount of the GRK5 co-immunoprecipitated with HDAC1 . H1, HDAC2; H2, HDAC2; H3, HDAC3; H8, HDAC8. These experiments were performed by Laura Johnson.

Although indicative, co-immunoprecipitation experiments do not confirm whether an interaction between two proteins is direct; it is possible that accessory proteins in the cell lysate bridge the gap between the proteins of interest. In order to ascertain whether GRK5 interacts directly with class I HDACs, HDAC1, HDAC3 and HDAC8, I performed direct binding assays using GST-HDACs purified from bacteria, which were immobilised on glutathione sepharose beads and incubated with GRK5 purified from SF9 cells. The samples were run on a SDS-PAGE gel and the amount of GRK5 binding directly to the fusion protein was detected by Western blotting using an anti-GRK4-6 antibody. GRK5 was pulled down with each of the three GST-HDACs but not with the GST negative control, thus suggesting that the kinase does indeed bind directly and specifically to HDAC1, HDAC3 and HDAC8 (Figure 3.2A). Quantification of multiple pull-down experiments shows GRK5 binds directly, and most strongly, to HDAC8 (Figure 3.2B), a pattern that is supported by the co-

immunoprecipitation data in Figure 3.1B. The weakest of the three interactions of GRK5 is that with HDAC3.

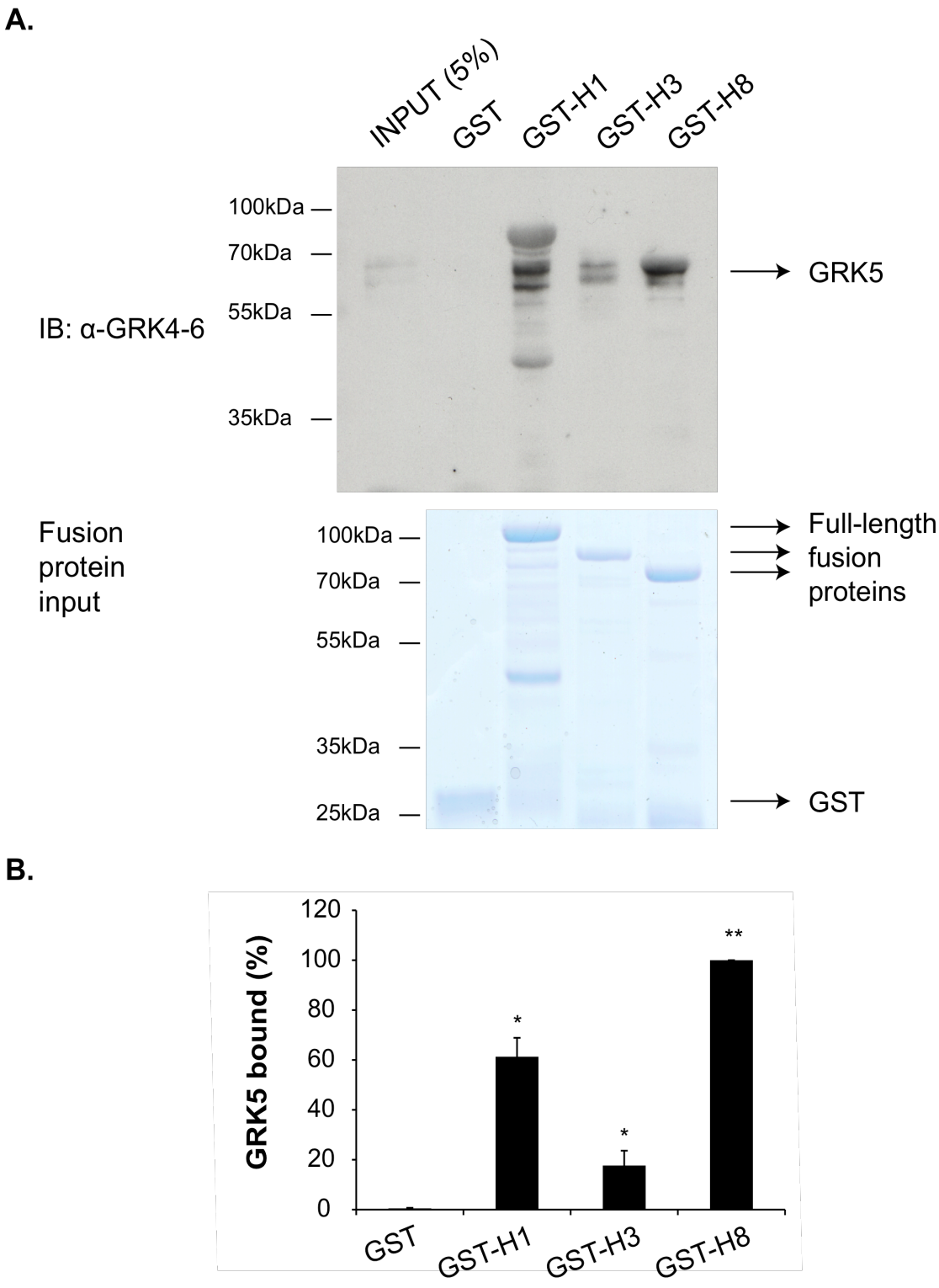


Figure 3.2 GRK5 binds class I HDACs directly

A. GST or GST-tagged class I HDACs were used to pull-down purified GRK5 from SF9 cells. Samples were run on SDS-PAGE gels and GRK5 binding detected by Western blotting, using an anti-GRK4-6 antibody (IB). The fusion protein inputs were checked by running equal amounts of GST and GST-HDACs on SDS-PAGE gels before Coomassie staining (Fusion protein input). The Western blot is a representative from 3 separate experiments. **B.** Quantification of A. The percentage of GRK5 bound to GST-HDAC per unit INPUT was quantified relative to the amount of GRK5 bound to GST and normalised to the percentage of GRK5 bound to HDAC8. Error bars represent standard error of the mean. *P<0.05, **P<0.005 relative to GST. H1 = HDAC1, H2 = HDAC2 etc.

3.2 GRK5 fails to interact with HDAC2

HDAC1 and HDAC2 are highly homologous proteins that share 85% sequence identity at the amino acid level, can heterodimerise and are often found in the same repressor complexes [167]. Several HDAC1/HDAC2 knockdown and knockout studies have highlighted redundant and compensatory roles for these HDACs. That being said, the global and targeted deletion of HDAC1 in mice results in early embryonic lethal death and a down-regulation of cellular HDAC activity is reported in HDAC1-null embryonic stem cells, despite the compensatory upregulation of HDAC2 [168]. HDAC1 therefore has certain autonomous roles that cannot be compensated for by HDAC2. Similarly, a role specific to HDAC2 has been reported with regards to apoptosis; selective inhibition of HDAC2 but not HDAC1 increased the sensitivity of breast cancer cells to tamoxifen treatment [169]. Co-immunoprecipitation of Flag-tagged HDACs with GRK5 identified an interaction specific to HDAC1 and not HDAC2 (section 3.1). To confirm the specificity of HDAC binding, I performed an immunofluorescence experiment to study whether

the cellular localisation of the HDAC could be influenced by GRK5, which would suggest a potential interaction between the HDAC and the kinase in a cellular setting.

The nuclear export of GRK5 can be stimulated in HEp2 cells by treatment with the calcium ionophore, A23187. GRK5 binds to Ca^{2+}/CaM with a high affinity at its N-terminus, which promotes the nuclear export of the kinase [10]. To extend this investigation, HEp2 cells were treated with A23187 and monitored as to whether HDAC nuclear export could be influenced by the presence of GRK5. When overexpressed individually in HEp2 cells, both HDAC1 and HDAC2 remain exclusively nuclear following A23187 treatment (Figure 3.3, panels a–d). Upon co-transfection of GRK5 with HDAC1, ionophore treatment promotes nuclear export of not only GRK5, (Figure 3.3, compare panels f to j and h to l) but also HDAC1 (Figure 3.3, panels e and i). In contrast, in cells expressing both GRK5 and HDAC2, HDAC2 remains exclusively nuclear following ionophore treatment (Figure 3.3, panels g and k). These data thus support the data obtained in the co-immunoprecipitation experiments, that GRK5 binds to HDAC1 but not HDAC2.

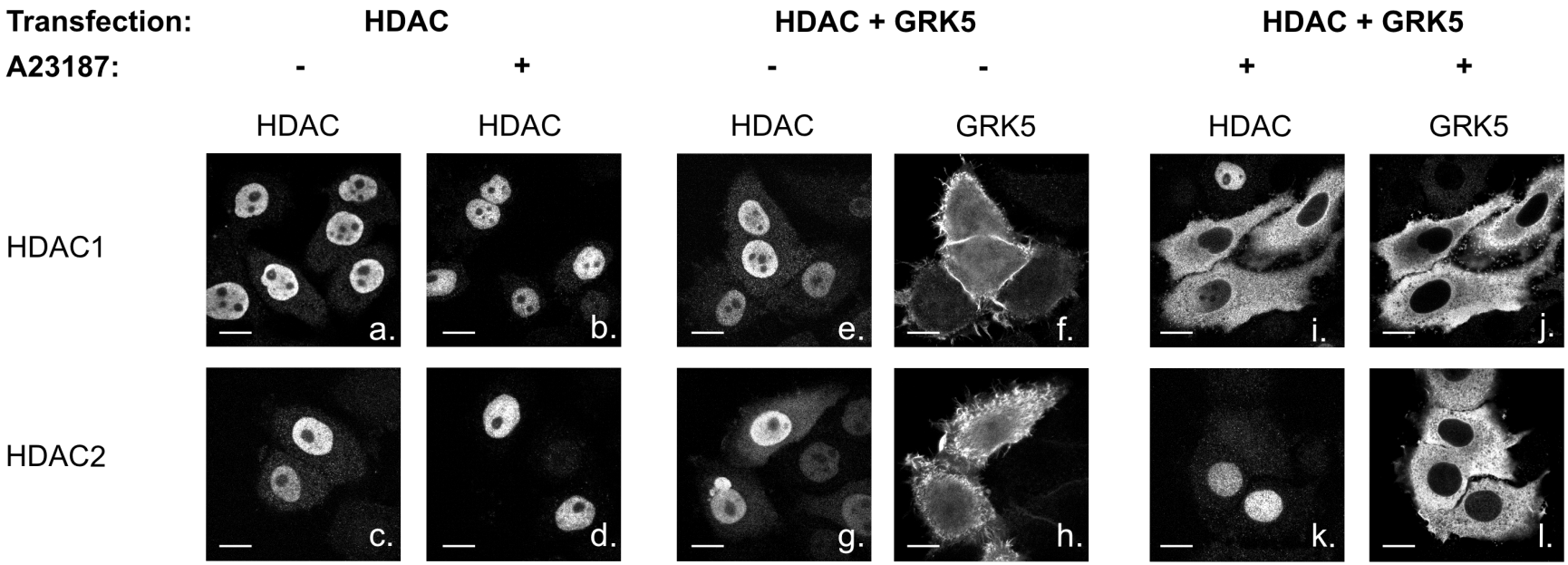


Figure 3.3 GRK5 stimulates nuclear export of HDAC1 but not HDAC2 following A23187 treatment

HEp2 cells were transfected as indicated and treated or not with the calcium ionophore, A23187 (+ A23187; 25 mM, 15 min). The subcellular distribution of GRK5 and HDAC1 or HDAC2 was subsequently visualised by indirect immunofluorescence using anti-GRK5 and anti-Flag antibodies to detect GRK5 and HDACs respectively. Scale bars 10 μm. These experiments were performed by Laura Johnson.

Data in Figure 3.2 shows the interaction between HDAC1 and GRK5 to be direct. As an additional test of the binding specificity of GRK5 with HDAC1 and HDAC2, I performed a direct binding assay with GST-GRK5 or GST (negative control) purified from bacteria and immobilised on glutathione sepharose beads, with which I incubated *in vitro* translated [^{35}S]-methionine labelled Flag-HDAC1 or Flag-HDAC2. The samples were washed, run on a SDS-PAGE gel and the gels dried down and exposed to X-ray film. Figure 3.4 confirms a direct interaction between GRK5 and HDAC1 and shows HDAC2 fails to interact directly with GRK5. Both the immunofluorescence (Figure 3.3) and pull-down data (Figure 3.4) confirm the findings from Figure 3.1 that indeed, GRK5 binds directly and specifically to HDAC1 but not HDAC2 in cells, cell lysates and *in vitro*.

Comparison of the HDAC1 and HDAC2 sequence alignments highlights a potential GRK5 binding site in the C-terminus of HDAC1. HDAC1 and HDAC2 share a 91% sequence identity in the combined N- and catalytic- domains, while the remaining approximately 40 amino acids of the C-termini are only around 34% identical [170]. Differences in HDAC1 and HDAC2 function may possibly be due to the interactions mediated by their C-termini, which, for HDAC1, is likely to include the binding site of GRK5.

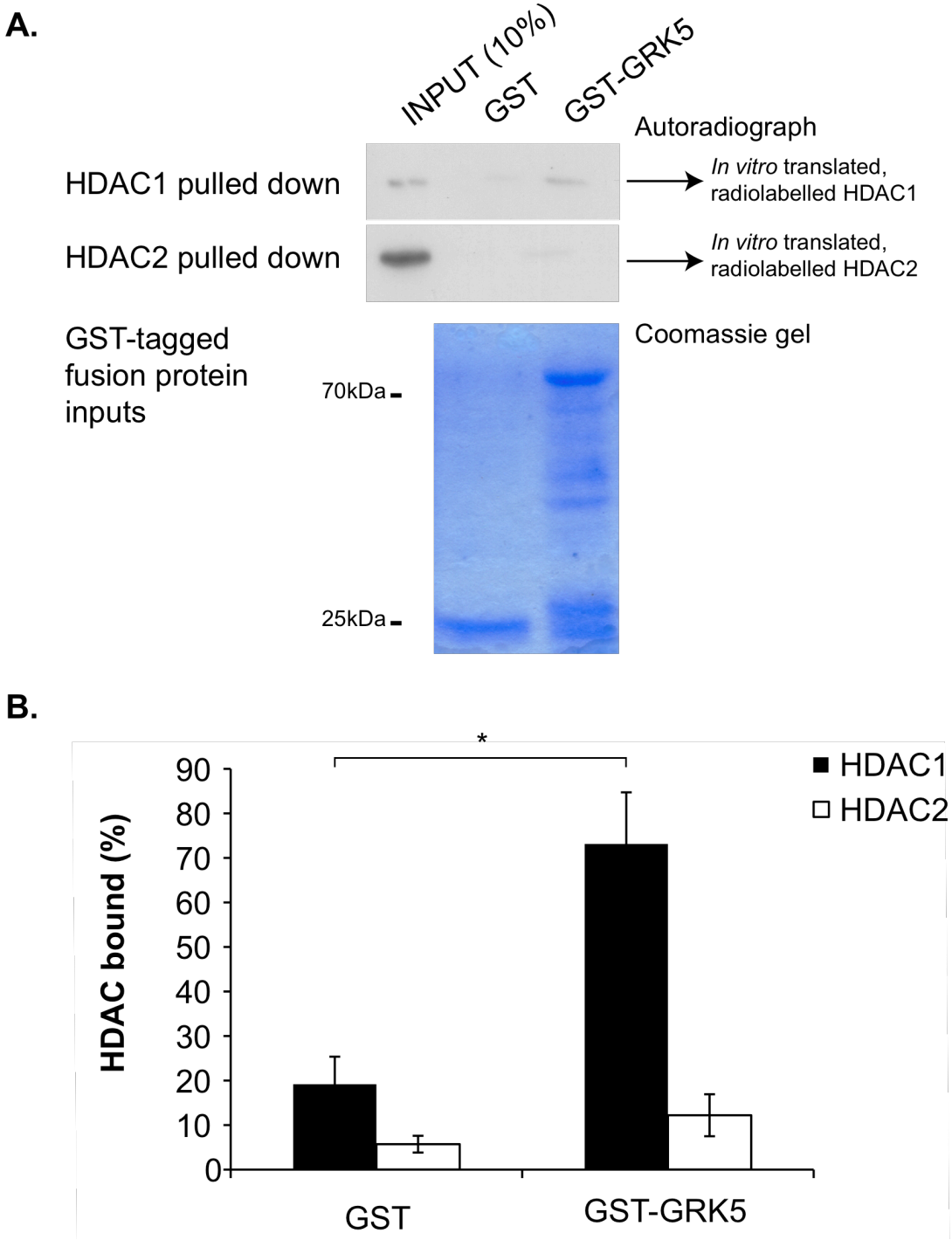


Figure 3.4 GRK5 does not bind HDAC2 directly

GST or GST-GRK5 was used to pull down *in vitro* translated, radiolabelled, Flag-tagged HDAC1 or HDAC2. Samples were run on a SDS-PAGE gel alongside 0.5 µl of HDAC1 or HDAC2 (INPUT). The fusion protein inputs were checked by running equal amounts on SDS-PAGE gels and detected by Coomassie staining. **A.** Representative Western blot from three separate

experiments. **B.** Quantification of A. HDAC binding (%) was quantified relative to INPUT. Error bars represent standard error of the mean from three separate experiments, *P<0.005.

3.3 GRK5 interacts directly with the C-terminus of Sin3A

HDACs are most commonly recognised for their role in regulating gene silencing, yet these proteins rarely function independently, as they lack a DNA binding domain. Instead, HDACs comprise the enzymatic components of multi-protein transcriptional repressor complexes. Indeed, HDAC1 and HDAC2 only display maximal catalytic activity when involved in such a complex [117, 130]. Numerous mammalian repressors have been characterised that recruit class I HDACs, as discussed in section 1.5 and include NuRD, REST/Co-REST, N-CoR/SMRT and Sin3 [130, 132-134]. Lan Ma's group performed a mass spectrometry screen in 2012 to identify novel interactors of GRK5. Flag-GRK5 was purified from the metastatic breast cancer cell line, MDA-MB-231, and samples run on SDS-PAGE gels. The screen identified and characterised damaged DNA-binding protein 1 (DDB1), part of an E3 ligase complex, as a major binding partner of GRK5. Another hit from this screen, but one which was not pursued by the group, was Sin3A [171]. Sin3A, a global regulator of transcription, incorporates HDAC1 and HDAC2 in its core repressor complex. In order to investigate whether the binding of class I HDACs to GRK5 could have a functional role, I decided to explore this potential GRK5/Sin3A interaction, to study whether GRK5 could play a role in regulating HDAC1 function within the context of this transcriptional repressor.

I extracted nuclei from HeLa cells, which are likely to be enriched in Sin3A, and compared the cellular localisation of endogenous GRK5 to that of Sin3A. Figure

3.5A shows, as would be expected, Sin3A and a few core components, HDAC1, HDAC2 and RBBP4, to be highly enriched in the nuclear fraction (N) compared to the whole cell lysate (W) and GRK5 has the same cellular distribution, while GRK2 is excluded from the nucleus. Considering the proteins of interest are all enriched in the nucleus, I used the HeLa nuclear extract to perform co-immunoprecipitation experiments to determine whether GRK5 may be part of the Sin3A repressor complex. Using an anti-Sin3A antibody, endogenous Sin3A was immunoprecipitated and GRK5 binding was detected by Western blotting using an anti-GRK5 antibody. Figure 3.5B shows endogenous GRK5 to interact with endogenous Sin3A.

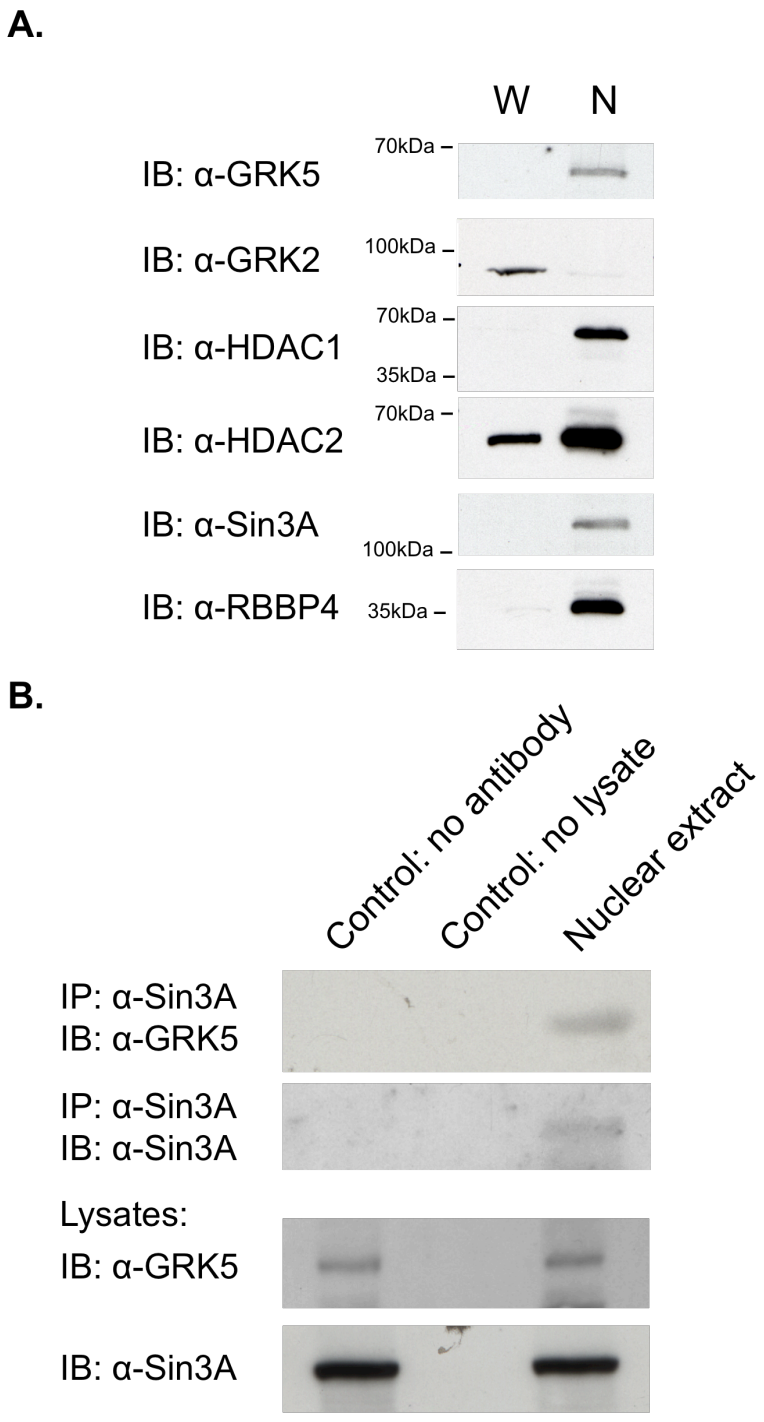


Figure 3.5 GRK5 interacts with Sin3A

A. HeLa whole cell lysates (W) and nuclear extracts (N) were immunoblotted (IB) for the proteins indicated. **B.** Co-immunoprecipitation of endogenous Sin3A and GRK5 from HeLa cell nuclear extracts. GRK5 binding to immunoprecipitated (IP) Sin3A was detected by

immunoblotting (IB). Endogenous protein expression was confirmed by Western blotting (lysates) using an anti-Sin3A and anti-GRK5 antibody respectively. The Western blot is a representative from 3 separate experiments.

Section 3.1 identified HDAC1 as a novel and direct interactor of GRK5. HDAC1 is a component of the Sin3A core complex and may therefore be responsible for recruiting GRK5 to Sin3A. In order to ascertain whether Sin3A is capable of interacting directly with GRK5 independently of HDAC1, I performed direct binding assays using immobilised GST-tagged Sin3A deletion mutants purified from bacteria and *in vitro* translated [^{35}S]-methionine labelled GRK5. This enabled not only the elucidation of whether the interaction between Sin3A and GRK5 is direct, but also enabled the broad scale mapping of the GRK5 interaction site on Sin3A. The Sin3A deletion mutants, as illustrated in Figure 3.6A, encompass each of the PAH domains, which mediate protein-protein interactions. The largest C-terminal fragment of Sin3A incorporates not only PAH4 but also the HID onto which the core complex assembles and the HCR, which is less well characterised. Data in Figure 3.6B demonstrates a direct interaction between the C terminal portion of Sin3A, encompassing residues 545–1157, and GRK5. Quantification of multiple experiments indicates binding to be exclusive to this portion of Sin3A (Figure 3.6C). The HCR and PAH4 domains of Sin3A are poorly characterised, with only a few protein interactions identified at these sites; most of the interactions that occur between residues 545–1157 involve the HID and the majority of the core Sin3A components bind to the region encompassing PAH3–HID [139]. GRK5 does not bind directly to the PAH3 domain but it is possible that, like HDAC1, GRK5 may bind to the HID of Sin3A, assembling in concert with, or in addition to, the Sin3A core complex proteins.

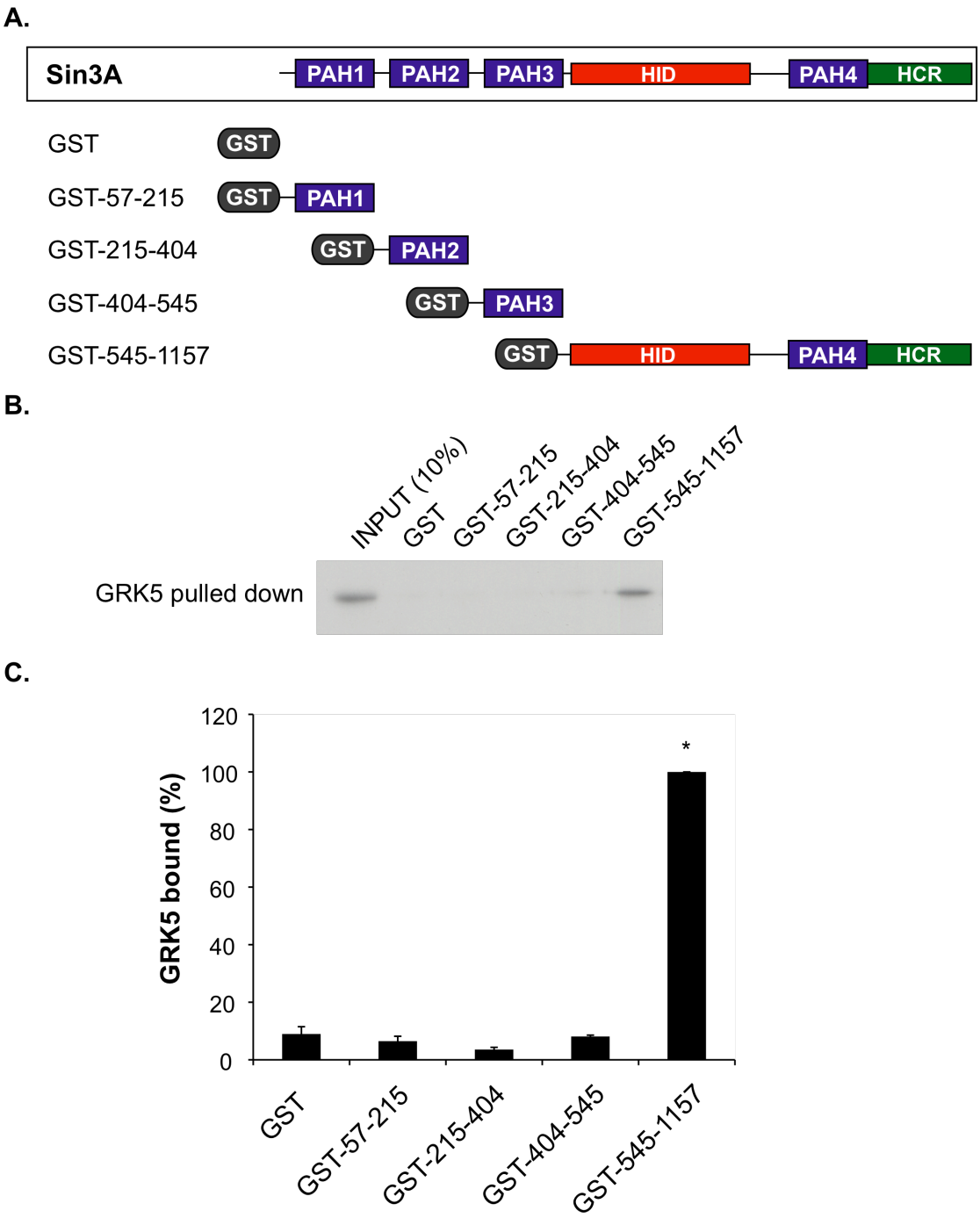


Figure 3.6 GRK5 interacts directly with the C-terminus of Sin3A

A. A schematic representative of the GST-Sin3A deletion mutants used in the pull-down experiments. **B.** *In vitro* translated radiolabelled GRK5 was incubated with GST or GST-Sin3A deletion mutants. Samples were run on a SDS-PAGE gel alongside 10% of the amount of *in vitro* translated GRK5 used in the experiment (INPUT). The fusion protein inputs were checked

by running equal amounts on SDS-PAGE gels and detected by Coomassie staining (not shown). **C.** Quantification of the amount of *in vitro* translated GRK5 directly bound to GST or GST-Sin3A deletion mutants relative to INPUT and normalised to the percentage GRK5 bound to GST-545-1157. Data is from three separate experiments and error bars represent standard error of the mean. *P<0.005 relative to GST.

3.4 Summary

This chapter describes the identification of novel GRK5 binding partners: class I HDACs and the repressor protein, Sin3A.

- In HeLa cells, GRK5 binds class I HDACs, HDAC1, HDAC3 and HDAC8 by co-immunoprecipitation, but does not interact with HDAC2 or class II HDACs.
- GRK5 binds HDAC1, HDAC3 and HDAC8 directly.
- Following A23187 treatment, GRK5 triggers the nuclear export of HDAC1 but not HDAC2 in HEp2 cells and binds to HDAC1 directly but not HDAC2 in *in vitro* direct binding assays. These data support the co-immunoprecipitation data in Figure 3.1 that implies GRK5 binds HDAC1 but not HDAC2.
- Endogenous GRK5 binds to the endogenous mammalian transcriptional repressor protein, Sin3A, by co-immunoprecipitation, in HeLa cells.
- GRK5 binds directly and exclusively to the C-terminus of Sin3A, which encompasses the HID, PAH4 and the HCR.

Chapter 4. Mapping the binding sites of HDAC1 and Sin3A on GRK5

4.1 GRK5 peptide arrays

The previous chapter saw the identification of class I HDACs, HDAC1, HDAC3 and HDAC8 and the transcriptional repressor protein, Sin3A, as direct binding partners of GRK5. One method of investigating the functional relevance of protein-protein interactions and thus the functional relevance of GRK5/class I HDAC and GRK5/Sin3A complex formation, is to study the physiological effects brought about by preventing such binding events. My results from chapter 3 suggest that GRK5 can interact both directly and indirectly with the Sin3A complex, via Sin3A itself but also via HDAC1. Preliminary *in vitro* mapping experiments or sequence analyses has enabled the broad-scale mapping of the GRK5 binding sites on Sin3A and HDAC1 respectively; direct binding assays identified GRK5 to bind to the C-terminus of Sin3A and the differential binding of HDAC1 and HDAC2 with GRK5 also suggests that GRK5 is likely to bind to the C-terminus of HDAC1, the region of the protein that varies most from HDAC2. To complement these findings and develop tools to ascertain the function of GRK5 as a binding partner of Sin3A and HDAC1, I wanted to map the binding sites of both proteins on GRK5.

The mutation of binding sites for novel binding partners on target proteins is often used as a means of investigating the physiological implication of the interaction. In order to ascertain the role of a novel binding partner in concert with a well-established protein complex, this new interactor should be the protein mutated in order to avoid disruption to the complex, which may have a variety of physiological

implications. Indeed, following the identification of growth factor receptor-bound protein 2 (Grb2) as a novel interactor of TNFR1, the binding site was mapped to the Src homology 3 domain on Grb2 and key residues mutated accordingly. Transfection of this TNFR1 binding deficient Grb2 mutant into HEK293 cells perturbed the TNFα-dependent activation of c-Raf-1, thus highlighting the specific importance of the interaction of TNFR1 with Grb2 in regulating this signalling pathway [172]. My aim, therefore, was to identify key residues on GRK5 that mediate the binding events with HDAC1 and Sin3A, the mutation of which would hopefully result in specific GRK5ΔHDAC1 and GRK5ΔSin3A binding deficient mutants without completely disrupting the Sin3A complex. These GRK5 mutants would then enable me to test the importance of these interactions in controlling GRK5-mediated cellular phenotypes.

SPOT™ immobilised peptide libraries of GRK5 were used in an attempt to map the binding sites of HDAC1 and Sin3A on the kinase. These peptide arrays are able to bind purified recombinant proteins and identify biologically active motifs. This technique, pioneered by Ronald Frank in the 1980s and first presented in 1990, involves multiple peptides synthesised and ‘spotted’ onto one membrane support. The technique utilises the concept that chemical reactions can proceed to completion only when enough reagent is used that can be taken up by the support material. Multiple reactions can therefore occur on one membrane as spot sizes are strictly controlled, such that peptides occupy distinct and isolated areas [173]. These membranes are used as tools to map binding sites, with one protein spotted as peptides onto the membrane, which is then overlaid with another protein of interest. For example, peptide arrays of β-arrestin 2 have been successfully utilised to identify the residues that mediate binding to family 4 cAMP-specific

phosphodiesterase, subfamily D, isoform 5 (PDE4D5) [174]. Moreover peptide array libraries of PDE4D5 showed that β-arrestin 2 and the receptor for activated C kinase 1 (RACK1) bind PDE4D5 at overlapping sites [175].

To increase the reliability of screening, the GRK5 arrays consist of overlapping 25-mer peptides each sequentially shifting along by five amino acids to span the entire GRK5 sequence (Figure 4.1A). Additionally, the arrays are duplicates, with the same peptide sequence printed twice on one array. The method used to detect sites of protein-protein interactions are much like a Western blot and illustrated schematically in Figure 4.1; the arrays are overlaid with purified GST-tagged proteins and sites of protein-protein interaction detected using an anti-GST antibody and the relevant secondary antibody (Figure 4.1B). The arrays are subsequently developed and imaged using X-ray film, with dark spots illustrating sites of positive protein-protein interactions (Figure 4.1B).

GRK5 peptides that replicate within and between experiments are subsequently selected for further mapping. These selected peptides are detected as strong spots on both sides of the duplicated array, as shown for peptide 3 on Figure 4.1B and similar patterns will have been detected in multiple experiments. Each amino acid of the chosen peptides are mutated sequentially to alanine and immobilised on an alanine scanning substitution array for incubation with target proteins (Figure 4.1C). Developing these arrays identifies residues important for mediating the binding events, as their mutation to alanine prevents these interactions from occurring, such that gaps appear in the developed array.

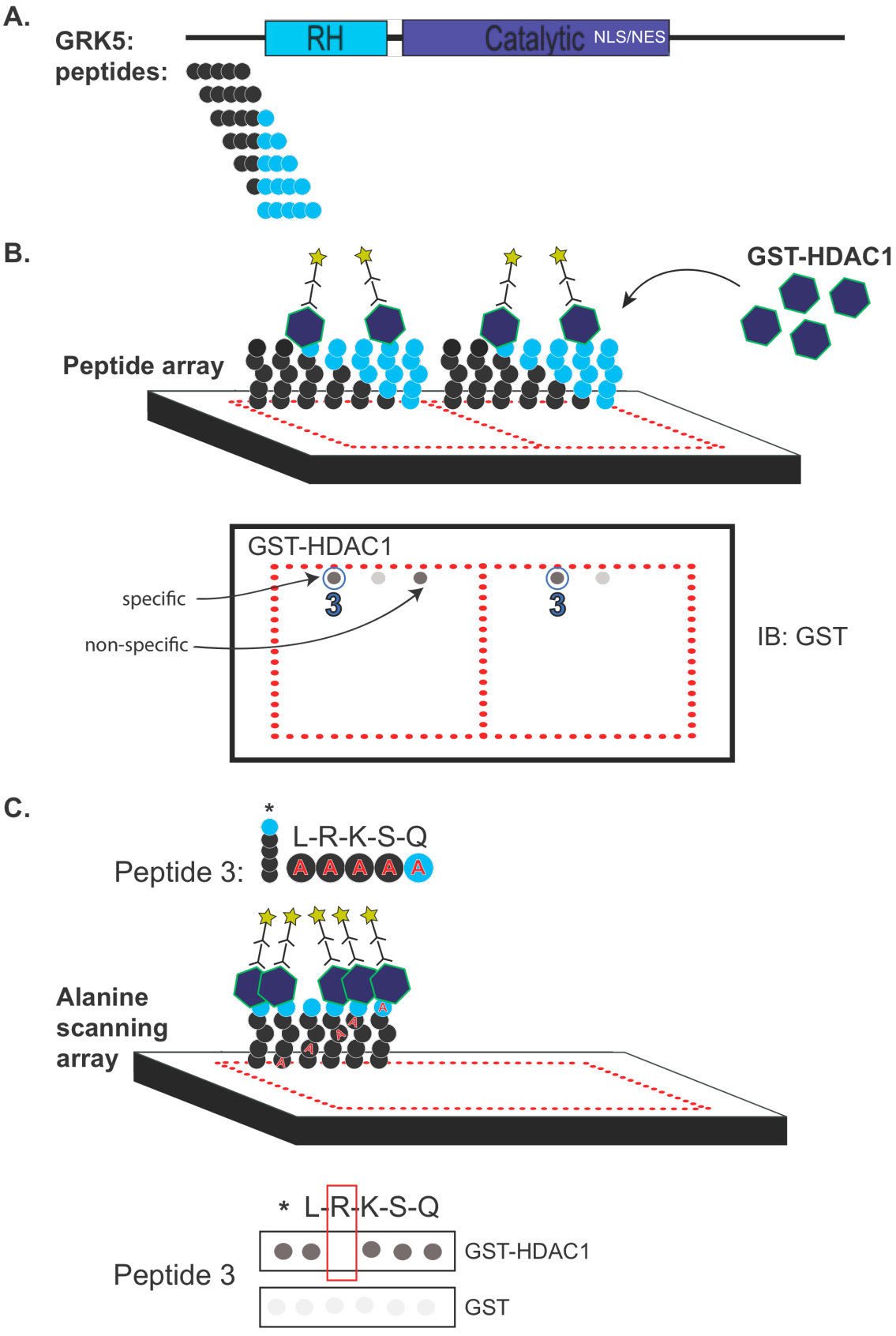

A.
GRK5:
peptides:
RH
Catalytic
NLS/NES
B.
GST-HDAC1
Peptide array
GST-HDAC1
specific
non-specific
3
3
IB: GST
C.
*
Peptide 3:
L-R-K-S-Q
A A A A A
Alanine
scanning
array
* L-R-K-S-Q
Peptide 3
GST-HDAC1
GST

Figure 4.1 Schematic representation of mapping protein binding sites using GRK5 peptide arrays

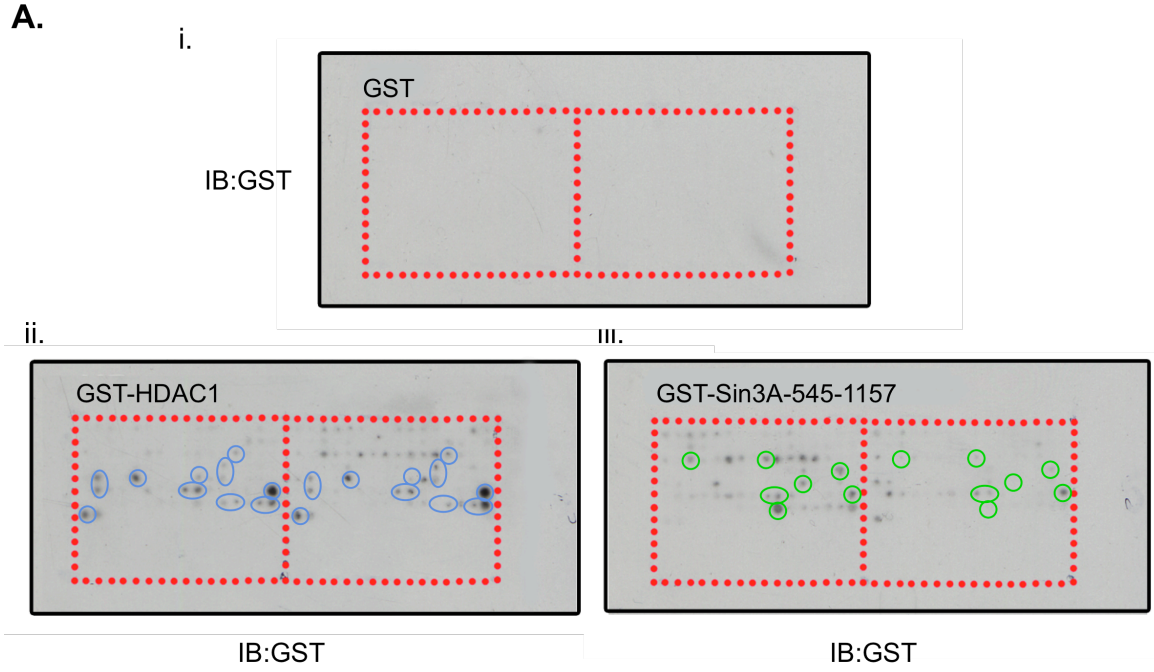
A. The GRK5 protein sequence is divided into 25-mer peptides, each overlapping by 5 amino acids. Each circle represents 5 amino acids. **B.** GRK5 peptide array. The GRK5 peptides are immobilised onto the array and incubated with target protein (GST-HDAC1 shown here for illustrative purposes), primary then secondary antibodies. The array is then developed and dark spots highlight sites of protein-protein interactions. Based on repeat data within and between experiments, selected spots are chosen that correspond to specific GRK5 peptide sequences, e.g. peptide 3, as shown in the schematic. **C**. Peptides corresponding to intensely stained and replicated spots are selected and used in an alanine scanning array. The array comprises firstly the full-length peptide (*) and then each residue in the chosen peptide is mutated sequentially to alanine. The method of incubation with target GST-proteins and detection using specific antibodies is as described in B. Developing the array highlights key residues needed for binding to the target protein.

4.2 Mapping the binding sites of HDAC1 and Sin3A on GRK5

GRK5 peptide arrays were incubated with GST-HDAC1 or GST-Sin3A-545-1157, the Sin3A fragment shown in section 3.3 to bind directly to GRK5, or with GST as a negative control. The arrays were then probed with an anti-GST antibody before the appropriate secondary antibody and developed as per a Western blot. In comparison to the GST control arrays, which remain largely blank (Figure 4.2Ai), the arrays probed with GST-HDAC1 (Figure 4.2Aii) or GST-Sin3A-545-1157 (Figure 4.2Aiii) display a series of dark spots, many of which are repeated on both sides of the array and in multiple experiments. Peptides that reproducibly bind GST-HDAC1 or GST-Sin3A are indicated in Figure 4.2Aii and iii by, respectively, blue and green circles. This colour coding holds true throughout, with blue and green corresponding to HDAC1 and Sin3A interacting peptides, respectively. The

location of these peptides within the sequence of GRK5 is shown in Figure 4.2B. The majority of these potential HDAC1 and Sin3A interacting peptides are located in the C-terminal portion of GRK5.

A.



B.

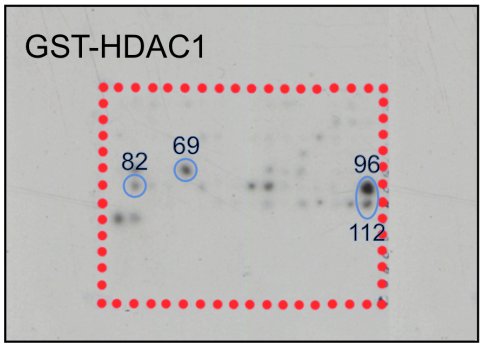
melenivant vllkareggg gkrkgkskkw keilkfphin
qcedlrrtid rdycslcdkq pvgrllfrqf cetrpglesy
iqfldsvaey evtpdeklge kgkeimtkyl tpkspvfitq
vgrdlvsqte ekllqkpcke lfsacvqsvh dylrgepfhe
yldsmyfdrf lqwkwlerqp vtkntfrqyr vlgkggfgev
cacqvratgk myackrlekk rikkrkgesm alnekqilek
vnsrfvvnla yayetkdalc lvltimnggd lkfhiynmgn
pgfeeeralf yaaeilcgle dlhhenivyr dlkpenilld
dyghirisdl glavkipegd lirgrvgtvg ymapevlnnq
ryglspdywg lgcliyemie gqspfrgrke kvkreevdrr
vleteevysh kfseeaksic kmlltkdakq rlgcqeegaa
evkrhpffrn mnfkrleagm ldppfvpdpr avyckdvldi
eqfstvkgvn ldhtdddfys kfstgsvpip wqsemietec
fkelnvfgph gtlspdlnrs hppeppkkgl lqrlfkrqhq
nnsksspnsk tsfnhhinsn hvssnstgss

Figure 4.2 Mapping the sites of HDAC1 and Sin3A interactions on GRK5

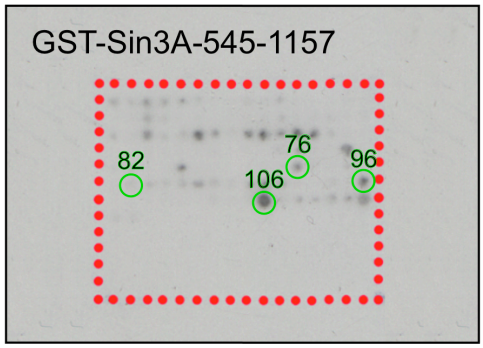
A. GRK5 peptide arrays were incubated with purified GST-HDAC1 (ii), GST-Sin3A-545-1157 (iii) or GST (control) (i) and probed with an anti-GST antibody to detect sites of protein-protein interaction. Dark spots on the arrays indicate sites of positive interactions. Spots detected repeatedly on multiple arrays within and between experiments are circled in blue or green to indicate HDAC1 or Sin3A interacting peptides respectively. **B.** The sequences of the peptides circled in A. are mapped onto the GRK5 peptide sequence, with potential HDAC1 interacting peptides highlighted in blue and Sin3A interacting peptides in green.

In an attempt to identify specific GRK5 residues important for binding HDAC1 and Sin3A, six peptides were incorporated into an alanine scanning substitution array. The peptides chosen reflect peptides repeatedly observed in multiple experiments to interact with target proteins. Unique to HDAC1 binding are peptides 69 and 112, highlighted in blue, and specific to Sin3A binding are peptides 76 and 106, which are highlighted in green (Figure 4.3A and B). Two additional peptides, 82 and 96, were also selected as being potentially involved in both HDAC1 and Sin3A binding events. The table in Figure 4.3C illustrates the sequences of the selected peptides and the location of the peptides within the GRK5 sequence is shown in Figure 4.4. The selected peptides span the C-terminal region of the catalytic domain as well as the C-terminus of the kinase.

A.



B.



C.

Peptide	Residues	Peptide sequence
69	341 - 355	L-I-R-G-R-V-G-T-V-G-Y-M-A-P-E-V-L-N-N-Q-R-Y-G-L-S
76	376 - 390	Y-E-M-I-E-G-Q-S-P-F-R-G-R-K-E-K-V-K-R-E-E-V-D-R-R
82	406 - 415	E-V-Y-S-H-K-F-S-E-E-A-K-S-I-C-K-M-L-L-T-K-D-A-K-Q
96	476 - 490	D-V-L-D-I-E-Q-F-S-T-V-K-G-V-N-L-D-H-T-D-D-D-F-Y-S
106	526 - 540	V-F-G-P-H-G-T-L-S-P-D-L-N-R-S-H-P-P-E-P-P-K-K-G-L
112	556 - 570	K-R-Q-H-Q-N-N-S-K-S-S-P-N-S-K-T-S-F-N-H-H-I-N-S-N

Figure 4.3 Selecting potential HDAC1 and Sin3A GRK5 interacting peptides

Six peptides that were detected on the GRK5 arrays from multiple experiments were selected for further mapping by alanine scanning. **A** and **B.** Selected peptides that interact with HDAC1 (A, blue) and Sin3A (B, green) are circled and numbered. **C.** The peptide sequence is given for each of the six selected GRK5 peptides and coloured according to whether these peptides mediate interactions with HDAC1 (blue) or Sin3A (green) or both (blue and green).

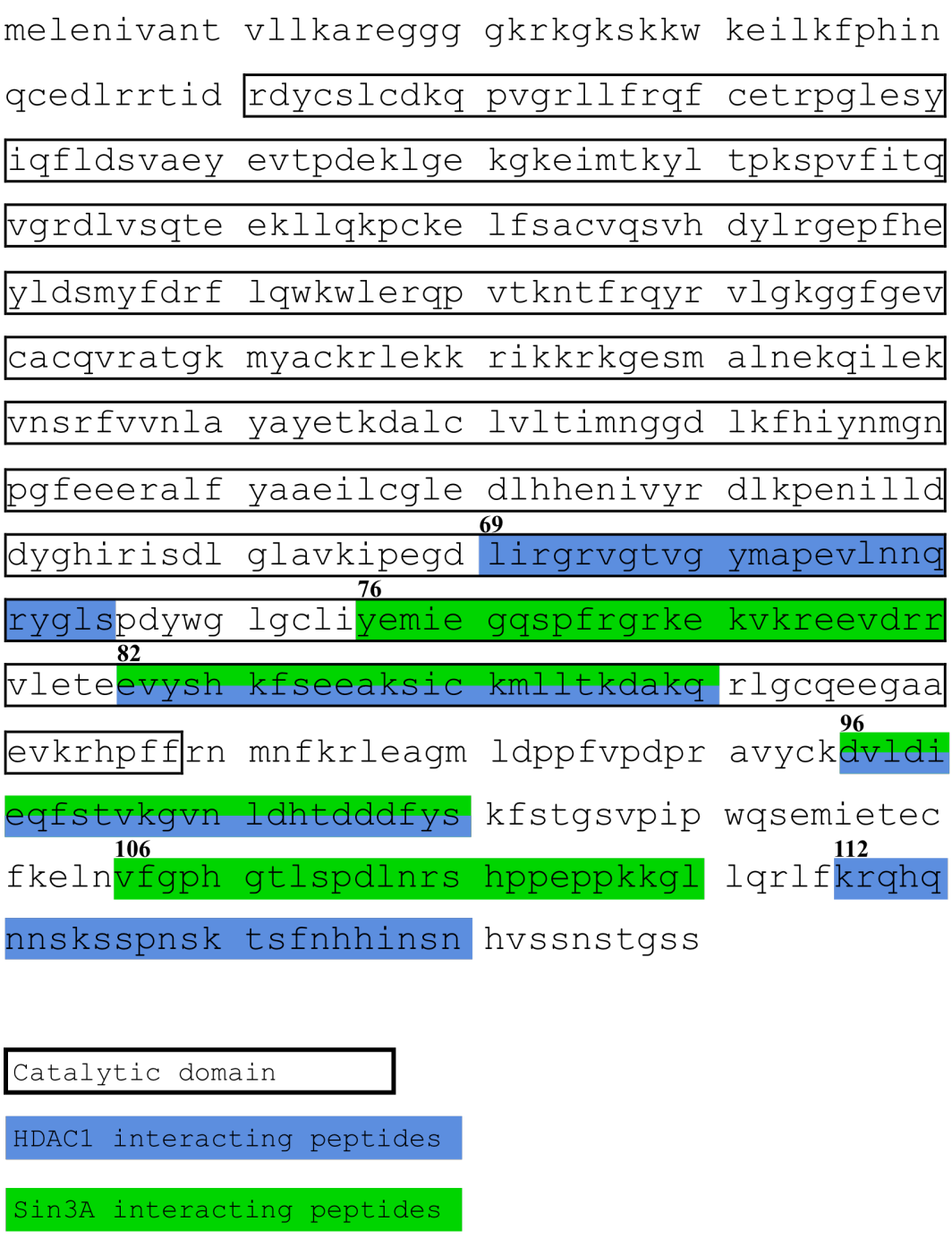


Figure 4.4 GRK5 protein sequence with potential interacting peptides

GRK5 peptides selected for the alanine scanning arrays are numbered and highlighted to show their potential interactions with HDAC1 (blue) and Sin3A (green) or both proteins (blue and green).

4.3 Identifying key residues for binding using Alanine scanning substitution arrays

Having identified six GRK5 peptides that were indicated by array analysis to contain HDAC1 and/or Sin3A binding sites, I then wanted to map these binding sites further and identify the specific residues required for these interactions. The selected peptides were used on an alanine scanning array, whereby each residue of the chosen peptide is mutated sequentially to alanine and these libraries were once again probed with target proteins, GST-HDAC1 or GST-Sin3A-545-1157. The first peptide on the array is the full-length GRK5 peptide, which is expected to interact with the target protein based on previous array analysis and is indicated in Figure 4.5 by an asterisk. Lack of binding to a mutated peptide, denoted by an absence of signal on the array, would therefore highlight an important residue for the binding event (Figure 4.1C).

GRK5 array results from Figure 4.3 suggest that peptides 69 and 112 are specific to mediating interactions with HDAC1. As shown in Figure 4.5A, mutation of each of the first 9 residues of peptide 69, Leu341–Val349, (L-I-R-G-R-V-G-T-V) to alanine increases the intensity of the interaction with GST-HDAC1, suggesting that this sequence may contain negative determinants in mediating the binding event. In contrast, mutating residues Gly350–Pro354 (G-Y-M-A-P) has little effect on binding compared to the full-length peptide. The intensity of the interaction with GST-HDAC1 is modestly reduced in comparison to the full-length peptide following the mutation of each of the residues Glu355–Arg361 (E-V-L-N-N-Q-R) to alanine, thus suggesting these amino acids may be of particular importance in mediating binding

to HDAC1. I decided to select the first two amino acids in this sequence, Glu355 and Val356, as residues to mutate in the full length GRK5 sequence to create a potential GRK5ΔHDAC1 binding mutant (Figure 4.5A, red box). The mutation of most of the residues in peptide 112 enhances the interaction between GRK5 and HDAC1 compared to the full-length peptide, with the possible exception of residues Gln358 and His359 (Figure 4.5A lower panel). Considering that mutating peptide 69 displays a reduced intensity of HDAC1 binding that spans more residues and is thus more likely to represent a bona fide binding site than peptide 112, peptide 112 was disregarded for any further analysis and only residues Glu355 and Val356 from peptide 69 were selected for mutation to create a potential GRK5ΔHDAC1 binding mutant.

Important for both HDAC1 and Sin3A binding are GRK5 peptides 82 and 96 (Figure 4.3). Binding of GRK5 peptide 82 to GST-HDAC1 is notably disrupted following the mutation of residues Lys411, Phe412, Glu414–Lys417 (E-E-A-K) and Lys421–Gln430 (M-L-L-T-K-D-A-K-Q) (Figure 4.5C). When overlaid with GST-Sin3A-545-1157 however, binding to even the full-length GRK5 peptide is very poor. The same is true for peptide 96. This may be due to a technical error with the alanine scanning array used in the Sin3A experiment, as only full-length peptide 76 has a significant interaction with GST-Sin3A-545-1157 (Figure 4.5B and C), which is not in keeping with data from Figure 4.3. I decided, therefore, to focus on the interactions of peptides 82 and 96 with GST-HDAC1 when selecting residues to mutate. Despite the reduced binding observed in the alanine scanning array of peptide 82, the alanine mutations of residues Asp485–Tyr489 (D-D-D-F-Y) in peptide 96 appeared to completely abolish binding to HDAC1 (Figure 4.5C).

Moreover, although the array may be unreliable, binding to Sin3A is also abolished following the mutation of the same residues, in comparison to the full-length peptide. I decided to select the first three residues in this sequence, Asp485–Asp487, to create a GRK5ΔHDAC1 and potentially a GRK5ΔHDAC1ΔSin3A binding mutant.

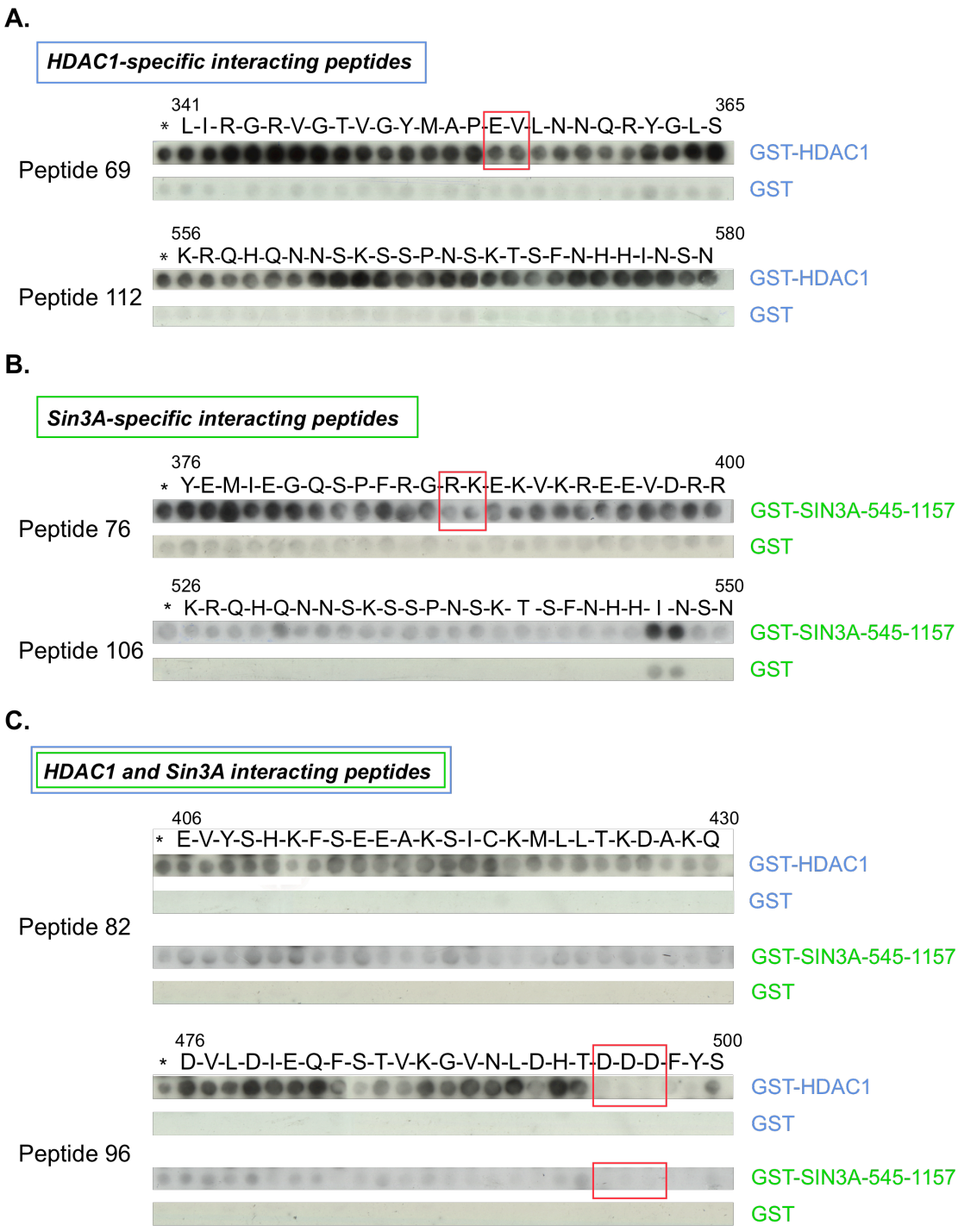


Figure 4.5 Mapping the sites of HDAC1 and Sin3A interactions on GRK5 using a GRK5 alanine scanning array

Individual residues in the selected peptides were mutated sequentially to alanine. * indicates the full length peptide with no mutations. The peptide sequence above the array indicates which

residue in that spot was mutated to alanine. GRK5 residues chosen to mutate are highlighted in red boxes. **A** and **B.** Arrays incubated with GST-HDAC1 or Sin3A are shown in blue and green respectively. **C.** Peptides 82 and 96 were incubated with both GST-HDAC1 and GST-Sin3A.

Specific to Sin3A binding are GRK5 peptides 76 and 106. As previously mentioned, on developing these alanine scanning arrays, full-length peptide 76 interacts stronger with Sin3A compared to peptide 106 (Figure 4.5, *). I therefore decided to focus on selecting residues to mutate from peptide 76. Sin3A binding to the peptide was reduced following the mutation of residues Ser383 and Pro384, the series Arg386–Asp398 (R-G-R-K-E-K-V-K-R-E-E-V-D), as well as the final residue in the peptide, Arg400. I decided to focus on the series of 12 residues whose interaction with Sin3A is reduced following mutation to alanine, as the likelihood is that multiple residues are involved in the binding event. In this sequence, residues Arg388 and Lys389 were noted as being the most important residues in the sequence for binding, as their mutation results in the weakest interaction with Sin3A-545-1157 (Figure 4.5B). These amino acids were therefore selected for mutation.

To summarise, of the six GRK5 peptides chosen for the alanine scanning arrays, residues from three peptides were selected for mutation, as highlighted in bold in Table 4.1. Out of the two peptides chosen for specificity towards HDAC1 binding, both peptides 69 and 112 contain residues that are negative determinants in the binding event, as strength of the interactions increases following their mutation to alanine. Peptide 69 contains more residues than peptide 112 that appear to be important for the binding event. The first two residues in this sequence, Glu355 and Val356, were selected for mutation. Considering full-length peptide 76 binds GST-Sin3A-545-1157 on the alanine scanning arrays stronger than full-length peptide

106, residues from this peptide were chosen for mutation. The first two amino acids in a series whose mutation to alanine reduces binding to Sin3A were selected: Arg388 and Lys389. When analysing the binding of HDAC1 and Sin3A to peptides 82 and 96, binding is abolished following the mutation of residues Asp485–Tyr489 (D-D-D-F-Y) of peptide 96. The first three amino acids in this sequence, Asp485–Asp487 were chosen for mutation. A summary of the residues selected for mutation is shown in Table 4.1.

Peptide	**Peptide sequence with mutated residues highlighted**	**Potential binding mutant**
69	L-I-R-G-R-V-G-T-V-G-Y-M-A-P-**E-V**-L-N-N-Q-R-Y-G-L-S	GRK5ΔHDAC1
76	Y-E-M-I-E-G-Q-S-P-F-R-G-**R-K**-E-K-V-K-R-E-E-V-D-R-R	GRK5ΔSin3A
96	D-V-L-D-I-E-Q-F-S-T-V-K-G-V-N-L-D-H-T-**D-D-D**-F-Y-S	GRK5ΔHDAC1Δ Sin3A

Table 4.1 GRK5 residues chosen for mutation based on alanine scanning array data

The GRK5 peptides selected for mutation are shown, along with the specific residues chosen to mutate (bold). The potential resultant GRK5 binding mutant is also given.

The residues selected for mutation were mapped onto the crystal structure of GRK6 in complex with AMP in order to confirm their location within the protein's tertiary structure. GRK5 and GRK6 share 70.1% amino acid sequence identity [24], such that the crystal structure of GRK6 was used to infer the position of the residues in GRK5, for which no crystal structure has been generated.

Figure 4.6 depicts the 3D ribbon structure (Figure 4.6A), and solvent exposed surface area (Figure 4.6B and C) of the GRK6 dimer. A sequence alignment of

GRK5 and GRK6 reveals residues Glu355 and Val356, a potential HDAC1 binding site on GRK5, are present at the same residue positions in GRK6. These residues are highlighted and labelled in blue in Figure 4.6A. The GRK5 region predicted to be involved in both HDAC1 and Sin3A interactions, Asp485–Asp486–Asp487, maps to a sequence on GRK6 ten residues along: Asp495–Gln496–Asp497. These residues are highlighted and labelled in sea green in Figure 4.6A and, as can be seen clearly in Figure 4.6B and C, lie on the surface of GRK6. The solvent exposed surface area of each of the residues, Asp495, Gln496 and Asp497 are 15.1, 107.4. and 108.0 Å, respectively, suggesting that Gln496 and Asp497 are extremely accessible to solvents and binding partners. In contrast, Glu355 and Val356 have a solvent accessible surface area of only 4.0 Å and 4.4 Å, respectively, and lie in a groove on the surface of GRK6 (Figure 4.6B and C). Residues Arg388 and Lys389 are not shown on the crystal structure but these residues are involved in the NLS of GRK5 and GRK6, which are known to be solvent exposed, as a GRK5ΔNLS mutant has been well characterised [10].

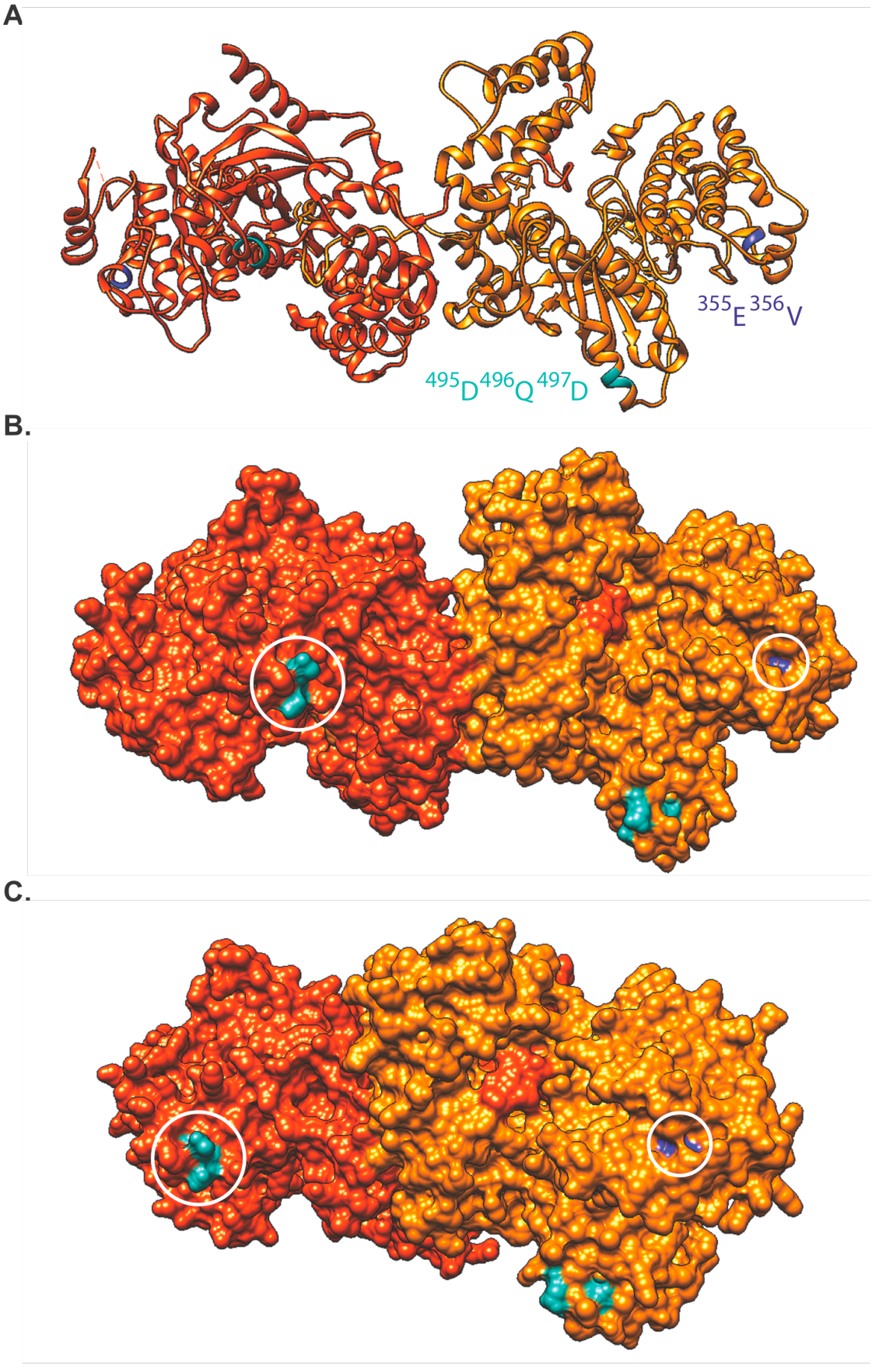

A
355E356V
495D496Q497D
B.
C.

Figure 4.6 GRK6 crystal structure with selected residues

The crystal structure of the GRK6 dimer with each monomer coloured in a different shade of orange. Residues 485–487, representing potential Sin3A and HDAC1 binding sites, are coloured in sea green. Residues 355 and 356, representing a potential HDAC1 binding site are coloured in blue. **A.** The 3D ribbon structure of GRK6 with labelled residues in the appropriate colours. **B.** The solvent exposed surface area of the GRK6 dimer is shown with circled residues corresponding to those labelled in A. **C.** The solvent exposed surface area of the GRK6 dimer is shown in a different orientation, highlighting the groove that contains residues 355 and 356.

While it is worthwhile knowing the location of the selected residues in the protein's tertiary structure, a potential conformational change may ensue upon interacting with a binding partner to alter the position of amino acids. The involvement of residues Glu355 and Val356 in mediating intermolecular interactions does, therefore, remain a possibility, particularly as these amino acids lie on the surface of GRK6. Furthermore, a GRK6 monomeric crystal structure may differ from the dimer crystal structure. While I do hypothesise GRK5 to dimerise in certain cellular settings, it may bind HDAC1 and Sin3A as a monomer and the selected residues may be more exposed in its monomeric form. Differences between GRK5 and GRK6 also mean that while the GRK6 crystal structure is informative, one cannot be certain that the same residues in GRK5 adopt a similar conformation. With this in mind I continued with this investigation and mutated the selected residues summarised in Table 4.1 to form potential GRK5 binding deficient mutants.

4.4 GRK5ΔNLS has a weaker interaction with HDAC1 and compared to wildtype GRK5

As described above and in Figure 4.5 and Table 4.1, three regions of the GRK5 protein sequence were identified as potential binding sites: Glu355 and Val356, and Arg388 and Lys389, specific to the binding of HDAC1 and Sin3A respectively, and Asp485–Asp487, which I predict to be important for both binding events. In order to test whether the residues identified are indeed important for HDAC1 and/or Sin3A binding, I mutated the GRK5 wildtype sequence to alanine at the selected sites and tested the ability of these mutants to bind to HDAC1 and Sin3A by performing direct binding assays. The mutated residues of a GRK5ΔNLS mutant include Arg388 and Lys389, the residues hypothesised to be necessary for the Sin3A/GRK5 interaction [10]. I therefore tested this GRK5ΔNLS mutant as a potential GRK5ΔSin3A binding deficient mutant and mutated Glu355 and Val356 and Asp485–Asp487 to alanine as a potential GRK5ΔHDAC1 (M1) and GRK5ΔHDAC1ΔSin3A binding mutants (M2–5) respectively, as shown in Table 4.2. To try and determine whether the three aspartic acid residues, Asp485–Asp487, work cooperatively or independently to mediate binding to HDAC1 and Sin3A, I decided to mutate each of these residues to alanine individually (M2–4) as well as together (M5) (Table 4.2).

Mutant name	Peptide	Mutation	Potential binding mutant
M1	69	E355A/V356A	GRK5ΔHDAC1
M2	96	D485A	GRK5ΔHDAC1ΔSin3A
M3	96	D486A	GRK5ΔHDAC1ΔSin3A
M4	96	D487A	GRK5ΔHDAC1ΔSin3A
M5	96	D485/486/487A	GRK5ΔHDAC1ΔSin3A

Table 4.2 GRK5 mutants

The GRK5 mutants are described, showing the mutations made and the peptides from which these residues were chosen, as a result of the peptide array experiments. Additionally, the potential GRK5 binding mutant generated from these mutations is also given.

I tested the ability of these GRK5 mutants to bind Sin3A and HDAC1 by *in vitro* translating the mutants with [^{35}S]-methionine, for use in direct binding assays with immobilised GST-HDAC1, GST-Sin3A-545-1157 or GST as a control (Figure 4.7). The potential GRK5ΔHDAC1 binding mutant, M1, behaves like wildtype GRK5 and binds similarly to HDAC1. I also tested this mutant for Sin3A binding and again, M1 behaves like wildtype kinase, as expected. The potential GRK5ΔHDAC1ΔSin3A mutants, M2, M3, and M5, bind HDAC1 and Sin3A to a similar degree as wildtype GRK5. From quantification of three independent experiments, mutant M4, encompassing a single alanine mutation at residue Asp487 (Table 4.2), has a stronger interaction with HDAC1 and Sin3A compared to wildtype GRK5 (Figure 4.7B). This aspartic acid residue may act as a negative determinant of HDAC1 and Sin3A binding when incorporated into the tertiary GRK5 structure, but this is likely to be is likely due to an experimental error from one ambiguous result.

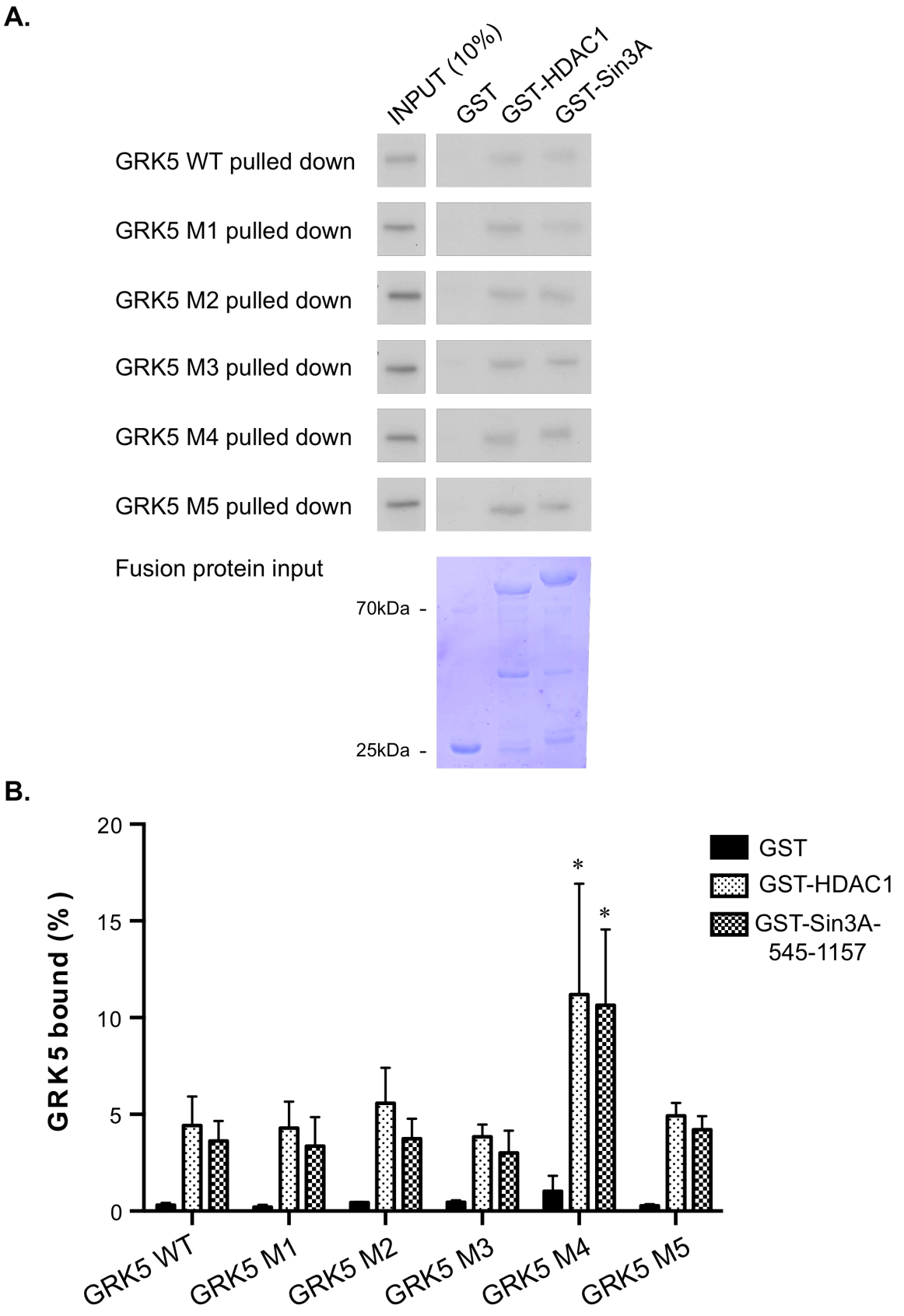

A.
INPUT (10%)
GST
GST-HDAC1
GST-Sin3A
GRK5 WT pulled down
GRK5 M1 pulled down
GRK5 M2 pulled down
GRK5 M3 pulled down
GRK5 M4 pulled down
GRK5 M5 pulled down
Fusion protein input
70kDa -
25kDa -
B.
GRK5 bound (%)
20
15
10
5
0
*
*
GST
GST-HDAC1
GST-Sin3A-545-1157
GRK5 WT
GRK5 M1
GRK5 M2
GRK5 M3
GRK5 M4
GRK5 M5

Figure 4.7 GRK5 mutants interact with HDAC1 and Sin3A

GST (control), GST-HDAC1 or GST-Sin3A-545-1157 were immobilised and incubated with *in vitro* translated, radiolabelled GRK5 wildtype (WT) or mutants (M1–5). Samples were run on SDS-PAGE gels, dried down and exposed to film. The fusion protein inputs were checked by running equal amounts of protein on SDS-PAGE gels before Coomassie staining. **A.** Representative Western blot of 3 separate experiments. **B.** Quantification of A. The bar graph shows percentage of GRK5 bound to fusion proteins per unit GRK5 INPUT. Error bars represent standard error of the mean from three separate experiments. *P<0.5 relative to GRK5 WT.

The GRK5ΔNLS mutant, tested as a potential GRK5ΔSin3A mutant, binds approximately 50% less well to both HDAC1 and Sin3A, compared to wildtype GRK5 (Figure 4.8B and C). The NLS of GRK5 encompasses residues Arg388–Glu395 (R-K-E-K-V-K-R-E), with arginine and lysine residues of this sequence mutated to form the GRK5ΔNLS construct (A-A-E-A-V-A-A-E), as shown in Figure 4.8A, where mutated residues are in red boxes. While the suspected residues that mediate the interaction with Sin3A, Arg388 and Lys389, are mutated in this GRK5ΔNLS mutant, we cannot rule out the possibility that the loss of binding to HDAC1 and Sin3A could be due to the additional mutations in the sequence, which exclude the kinase from the nucleus.

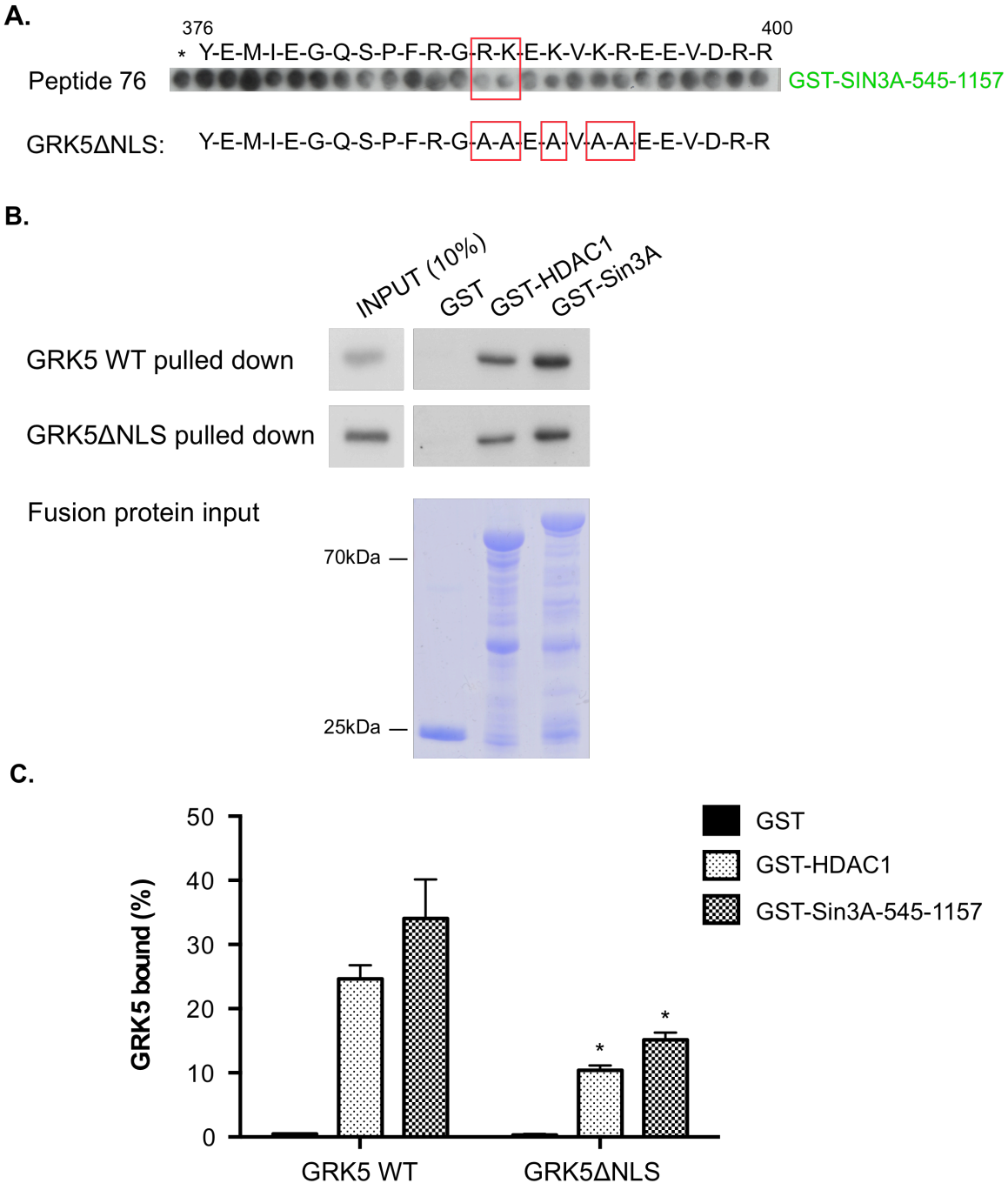


Figure 4.8 GRK5ΔNLS interacts with HDAC1 and Sin3A

A. Comparison of residues mutated in the GRK5ΔNLS mutant (bottom line) to the residues selected to mutate from the alanine scanning array of peptide 76, to potentially create a Sin3A binding deficient mutant (top line). Mutated residues are boxed in red. **B.** GST (control), GST-HDAC1 or GST-Sin3A-545-1157 were immobilised and incubated with *in vitro* translated,

radiolabelled GRK5 wildtype (WT) or NLS mutant (GRK5ΔNLS). Samples were run on SDS-PAGE gels, dried down and exposed to film. The fusion protein inputs were checked by running equal amounts of protein on SDS-PAGE gels before Coomassie staining. Shown is a representative Western blot of 4 separate experiments. **C.** Quantification of A. The bar graph shows percentage of GRK5 bound to fusion proteins per unit GRK5 INPUT. Error bars represent standard error of the mean from four separate experiments. *P<0.05 relative to GRK5.

4.5 Summary

In this chapter I attempted to map the HDAC1 and Sin3A binding sites on GRK5.

- Using GRK5 peptide arrays comprising 25mer immobilised peptides, I attempted to identify specific GRK5 peptides that mediate binding to HDAC1 and Sin3A.
- Following the analysis of arrays developed from multiple experiments, specific peptides were selected based on repeat binding patterns to the target proteins, as shown in Table 4.3. These residues were incorporated into alanine scanning arrays and selected residues chosen to mutate.

Peptide	**Potentially binds to**	**Alanine scanning arrays**	**Residues to mutate**
69	HDAC1	More residues act as positive binding determinants than peptide 112.	Glu355 and Val356
112	HDAC1	Many residues act as negative determinants.	
76	Sin3A	Full-length peptide binds Sin3A stronger than peptide 106.	Arg388 and Lys389

			(GRK5ΔNLS)
106	Sin3A	Full-length peptide did not strongly interact with Sin3A.	
82	HDAC1 and Sin3A	Loss of binding is not as great as that shown in peptide 96.	
96	HDAC1 and Sin3A	Mutation of certain residues totally abolishes binding to target proteins.	Asp485–Asp487

Table 4.3 Summary of peptide array results

The table describes the peptides chosen for use in the alanine scanning arrays and summarises the results of these arrays. Residues from selected peptides chosen to mutate are also displayed.

- Mapping the selected residues onto the crystal structure of GRK6 shows Asp485–Asp487 are located on the surface of the kinase, accessible to solvent and binding partners. Residues Glu355 and Val356 lie in a groove on the surface of GRK6 and are unlikely to mediate intermolecular interactions in the given conformation, but conformational changes that occur upon protein binding and discrepancies between the GRK5 and GRK6 amino acid sequence, and thus potentially their tertiary structures, means these residues may still be involved in mediating protein-protein interactions.
- The following GRK5 mutants were made based on analysis of the alanine scans: E355A/V356A, D485A, D486A, D487A and D485/486/487A, which are referred to as mutants M1–M5, as shown in Table 4.2. None of the mutants disrupt binding to the fusion proteins compared to wildtype GRK5, as shown by direct binding assays.

- Mutant M4, D487A, displayed enhanced binding capabilities to both HDAC1 and Sin3A.
- A pre-existing GRK5ΔNLS mutant encompassing the R388A/K389A mutation was tested as a potential GRK5ΔSin3A binding mutant. Indeed, binding of the GRK5ΔNLS to both HDAC1 and Sin3A was perturbed in comparison to wildtype GRK5.
- Despite this result, the GRK5ΔNLS mutant will not be used to further investigate a physiological role for the GRK5/Sin3A or GRK5/HDAC1 interactions, as this mutant is excluded from the nucleus. Any functional differences observed with this mutant in comparison to wildtype GRK5 cannot, therefore, be solely attributed to perturbing the protein-protein interactions between GRK5ΔNLS and Sin3A or HDAC1, but may also be due to mislocalisation of GRK5.

In light of my inability to identify GRK5 mutants deficient in HDAC1 and/or Sin3A binding, I decided to investigate a functional role for nuclear GRK5 in coordination with HDAC1 and the Sin3A complex through the use of HDAC and Sin3A inhibitors. The following chapter describes these findings in detail.

Chapter 5. GRK5 represses Bcl-2 transcription to increase the sensitivity of HT-29 cells to chemotherapeutic agents

GRKs play a part in disease pathogenesis through their role as GPCR kinases. As increasing numbers of non-receptor GRK substrates and binding partners are being identified, their effector portfolio in both physiological and pathophysiological processes has greatly expanded beyond that of receptor desensitisation. Indeed, nuclear GRK5 has been implicated in pathological cardiac hypertrophy through, at least in part, relieving the inhibition of the pro-hypertrophic transcription factor, MEF2, by acting as an HDAC5 kinase [81].

Chapter 3 saw the identification of class I HDACs and Sin3A as novel GRK5 binding partners. I outlined in chapter 4 a method used to attempt to map the binding sites for these proteins on GRK5, with the aim of making GRK5 binding deficient mutants. Identification of these novel GRK5 binding partners suggests a role for the kinase in transcriptional repression, but the only identified HDAC1 and Sin3A binding deficient mutant, GRK5ΔNLS, is exclusively cytoplasmic. Disrupted protein binding cannot be distinguished from the mutant's mislocalisation, thus preventing further use of the GRK5ΔNLS construct in investigating a functional role for the GRK5/HDAC1 and GRK5/Sin3A interactions. An alternative approach to probing the physiological implications of protein-protein interactions is through the use of signalling inhibitors and protein knockdown. Both of these techniques were employed to investigate a potential role for GRK5, in concert with HDAC1 and Sin3A, in a model of colon cancer.

The aberrant growth of cancerous cells is due to the accumulation of multiple genetic mutations that render cells resistant to cell cycle control checkpoints and many environmental cues. The progression of tissues from benign to malignant involves the deregulation of multiple genes and altered gene expression accordingly. Aberrant activation or expression of GPCRs is associated with tumour growth and cancer progression. GRK5 is implicated in cancer pathogenesis not only due to its role as a GPCR kinase, but also in a more direct manner, via its ability to phosphorylate nuclear substrates, including p53. GRK5 is involved in the pathogenesis of GBM, thyroid, prostate and colon cancer, as discussed in section 1.4.

Colon cancer is one of the leading causes of cancer deaths worldwide and GRK5 has recently been implicated in the disease pathogenesis: a whole genome association study identified a SNP in the intron of GRK5 to be associated with a 35% increased risk of developing CRC [Dr Stephen Gruber, Patent publication number: WO2009046422 A2]. Whether this SNP affects the expression levels of GRK5 is yet to be determined, but a recent microarray study of gene expression profiles of patients with colorectal liver metastases identified GRK5 as being significantly under expressed in higher-risk patients [Chung, J., 2013; personal communication]. GRK5 expression is positively regulated by the tumour suppressor protein, tazarotene-induced gene 1 (TIG1), which inhibits cell growth and is downregulated in metastatic colon cancer cells. Furthermore, GRK5 suppression is responsible for TIG1-mediated growth regulation in colon cancer cells [176]. There is, therefore, potential for GRK5 to act as a biomarker for CRC, with levels of the kinase possibly indicating the stage of disease.

5.1 GRK5 levels vary across cancer cell lines

There is considerable evidence that GRK5 is downregulated in colon cancer. To investigate whether this down-regulation contributes to disease progression and to investigate a potential function of the GRK5/HDAC1 and GRK5/Sin3A interactions in this process, I sought a model cellular system to use. I screened a range of cancer cell lines to compare how GRK5 protein expression levels vary in different cancers and whether kinase levels in colon cancer are notably lower. GRK5 is expressed in the cervical cancer cell line, HeLa, as well as in the prostate cancer cell line, PC3, as mentioned in sections 3.3 and 1.4.3.1 respectively. In addition to these two cell lines, three ovarian cancer cell lines, OVCAR3, IGROV-1 and A2780 as well as two colon cancer cell lines, SW620 and HT-29, were tested for endogenous GRK5 protein expression levels.

Cell nuclear extracts were prepared from the range of cancer cell lines and run together with the whole cell lysate on a SDS-PAGE gel and probed with an anti-GRK5 antibody. Figure 5.1 shows GRK5 to be enriched in nuclear extracts but is barely detectable in the remaining cell lysate. Furthermore, nuclear GRK5 levels vary greatly between different cancers cell lines. For comparison purposes, the amount of nuclear GRK5 per unit actin expression, as shown in the bar graph of Figure 5.1, is arbitrarily set to 100% for HeLa cells. All three ovarian cancer cell lines have the highest nuclear GRK5 expression levels and the lowest expression is found in colon cancer cells, HT-29 and SW620. These cell lines are derived from colon adenocarcinomas; HT-29 cells are primary colon epithelial cells from a grade II tumour, localised only to the bowl and are yet to metastasise. By contrast, SW620 are metastatic colon cancer cells taken from lymph nodes. Relative to

HeLa cells, nuclear GRK5 protein levels in HT-29 and SW620 cells are 12.1% ± 2.1 and 14.0% ± 0.2 respectively (Figure 5.1B). OVCAR3 cells have approximately 10-fold more GRK5 compared to the colon cancer cells. In contrast to GRK5, which is enriched in the nucleus, GRK2 is largely cytoplasmic (Figure 5.1A). This is hardly surprising, as the GRK2 subfamily lack the NLS. The detection of small amounts of nuclear GRK2 could result from cytoplasmic contamination of the nuclear extract. Considering both colon cancer cell lines have by far the lowest GRK5 protein levels, these data support the hypothesis that GRK5 levels may be downregulated in colon cancer. HT-29 cells were chosen as a model cell line, as they are widely used as an *in vitro* model of colon cancer. As described, these cells are derived from a lower grade colon cancer tumour compared to SW620, yet the cells already show a resistance to apoptosis, suggesting a trend towards metastasis [177]. Using HT-29 cells for further studies rather than SW620 cells could identify whether GRK5 is having a causative role at the earlier stages of disease pathogenesis and may even highlight a potential role for GRK5 as a colon cancer biomarker.

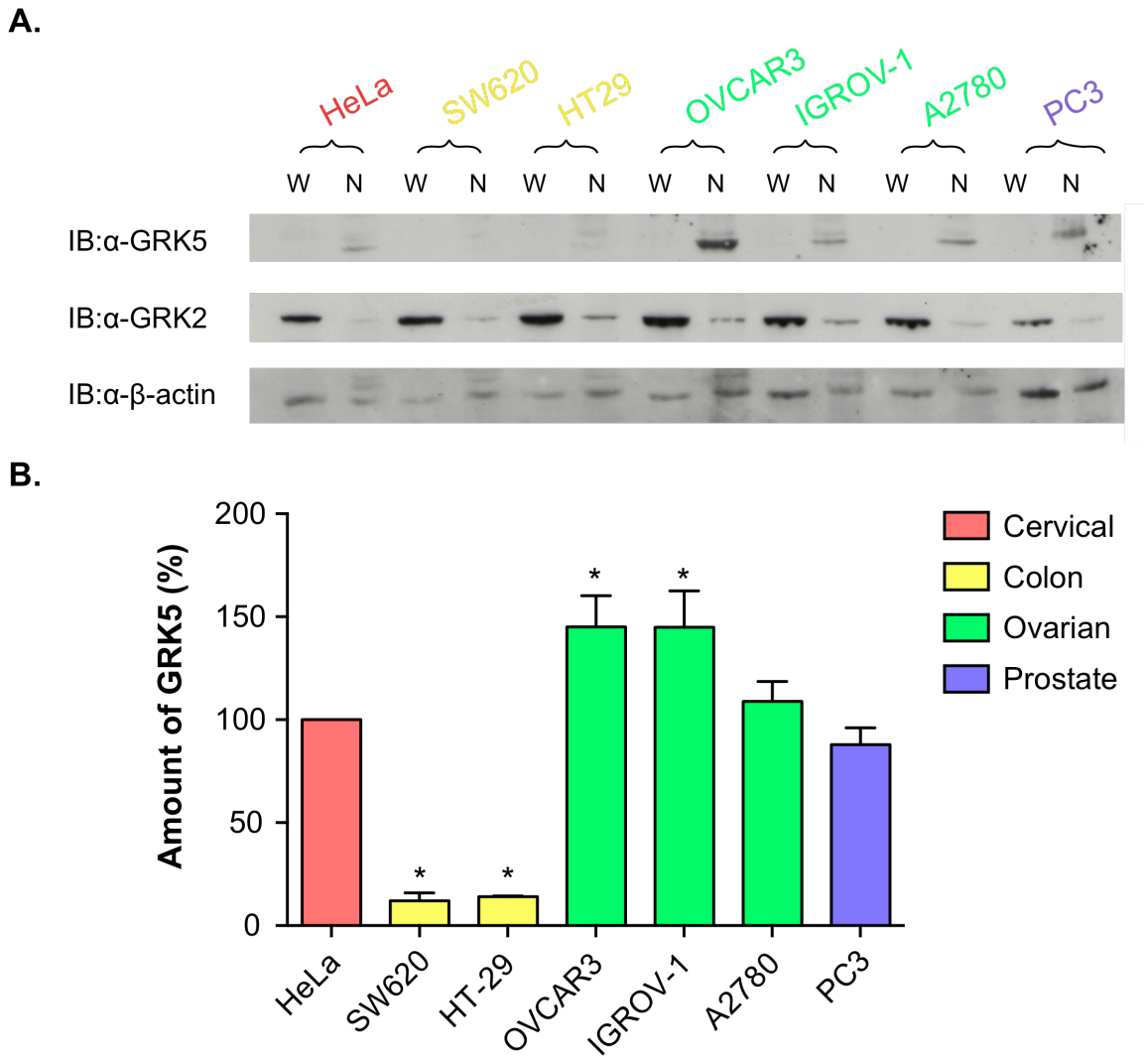


Figure 5.1 GRK5 levels are lowest in colon cancer

A. Nuclear extracts were prepared from a range of cancer cell lines. The coloured fonts indicate the type of cancer with red (HeLa) as cervical cancer, yellow (SW620 and HT-29) as colon cancer, green (OVCAR3, IGROV-1 and A2780) as ovarian cancer and purple (PC3) as prostate cancer. Nuclear extracts (N) and whole cell lysates (W) were run on SDS-PAGE gels and immunoblotted (IB) with an anti-GRK5 or anti-GRK2 antibody and an anti-β-actin antibody, as a loading control. **B.** Quantification of mean levels of GRK5 in nuclear extracts relative to β-actin and normalised to GRK5 levels in HeLa cells. Error bars are standard deviation of the mean from three separate experiments. *P<0.05 relative to the amount of GRK5 in HeLa cells, which is arbitrarily set to 100%.

5.2 GRK5 represses Bcl-2 in a class I HDAC-dependent manner

Levels of the anti-apoptotic protein, Bcl-2, are upregulated during the early stages of progression of an adenomatous polyp to a colorectal carcinoma. GRK5 has been shown to negatively regulate Bcl-2 transcription in SHSY5Y cells [80]. It is therefore feasible that GRK5 may be playing a causative role in colon cancer progression to a metastatic state; down-regulation of the kinase may relieve Bcl-2 inhibition and render cells resistant to apoptosis, thus accelerating cancer cell growth. I overexpressed increasing amounts of GRK5 in HT-29 cells and by Western blotting, probing with an anti-Bcl-2 antibody, I detected whether GRK5 could alter Bcl-2 protein levels. Figure 5.2A indicates that indeed, GRK5 and Bcl-2 protein levels have a reciprocal relationship; increasing GRK5 expression levels causes a simultaneous reduction in Bcl-2 protein by up to 54% ± 7.02 ($n = 3$; $P<0.005$) (Figure 5.2B). In an initial experiment, knocking-down endogenous GRK5 by transfecting a GRK5 small inhibiting RNA (siRNA) into HT-29 cells causes the opposite pattern and results in a significant increase in Bcl-2 protein levels by 70.8% (Figure 5.2C). These data therefore suggest that GRK5 does, indeed, negatively regulate Bcl-2 protein levels.

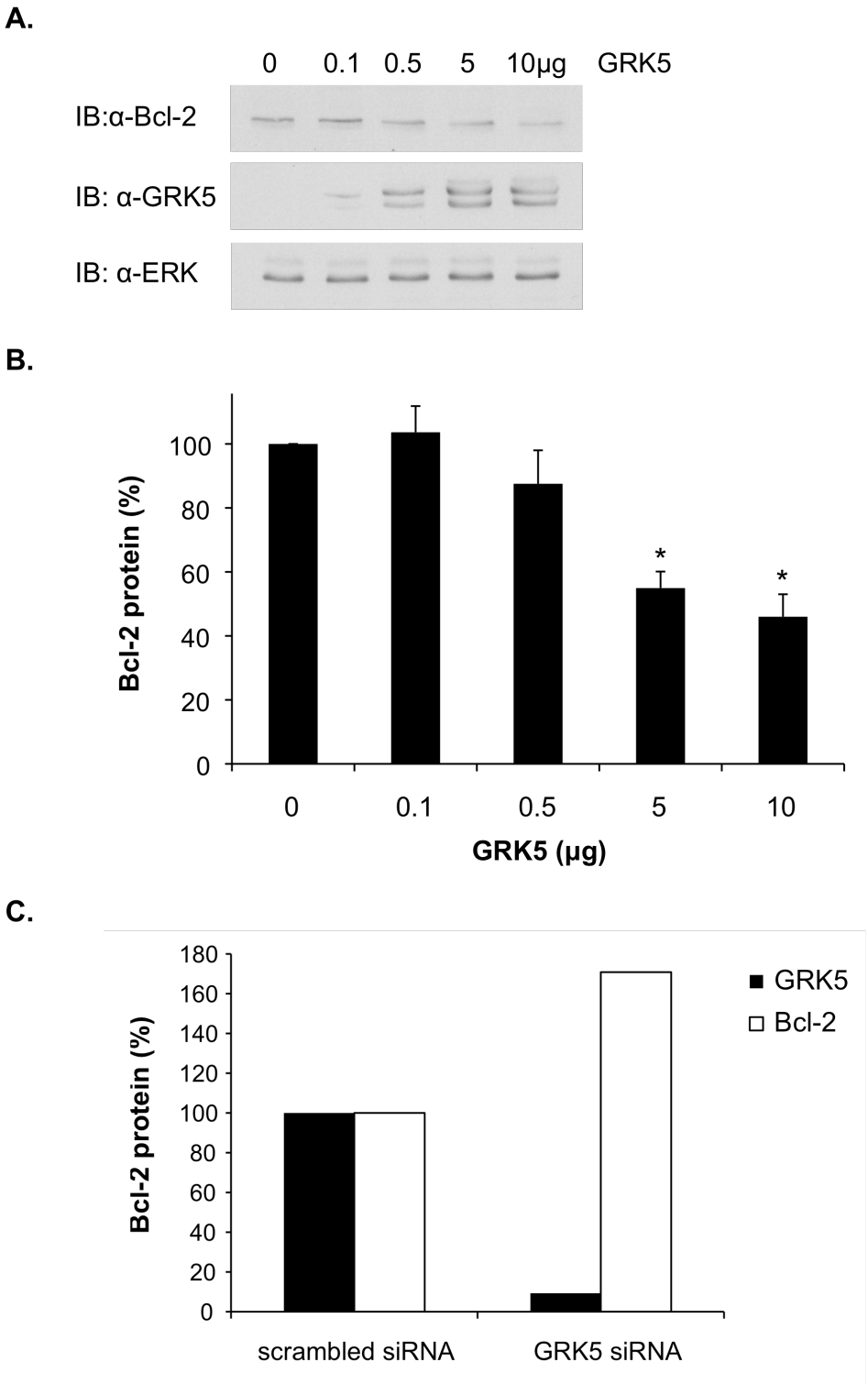


Figure 5.2 GRK5 reduces Bcl-2 protein levels in HT-29 cells

A. HT-29 cells were transfected with increasing amounts of GRK5 and Bcl-2 protein was detected by Western blot with an anti-GRK5 and anti-Bcl-2 antibody respectively. Endogenous

ERK expression was immunoblotted (IB) as a control. The Western blot shown is a representative from 3 separate experiments. **B.** Quantification of A. The bar graph represents average Bcl-2 levels from three separate experiments normalised relative to ERK expression levels. Error bars represent standard error of the mean from three separate experiments; *P<0.005 relative to 0 µg GRK5 transfected. **C**. HT-29 cells were transfected with a GRK5 siRNA or a scrambled control and 48 hours later cells were harvested and lysates run on SDS-PAGE gels to detect GRK5 and Bcl-2 protein expression levels. N = 1.

In order to investigate the mechanism by which GRK5 is regulating the levels of Bcl-2 expression, I treated HT-29 cells at the time of transfection with a pan-HDAC inhibitor, NaB, or a class I specific HDAC inhibitor, MS-275, or DMSO as a control. Treatment with either inhibitor abolishes the GRK5-dependent reduction of Bcl-2 protein levels, as shown in Figure 5.3, and in fact causes a marginal but significant increase in protein levels compared to controls. This suggests that the regulation of Bcl-2 by GRK5 is HDAC dependent. Data in chapter 3 identified class I HDACs as novel binding partners of GRK5 and, as shown in Figure 5.3, class I HDAC inhibitor treatment abolishes the GRK5-mediated regulation of Bcl-2 expression. The regulation of Bcl-2 by GRK5 is therefore likely to be class I HDAC-dependent, the first functional connection reported between GRK5 and class I HDACs.

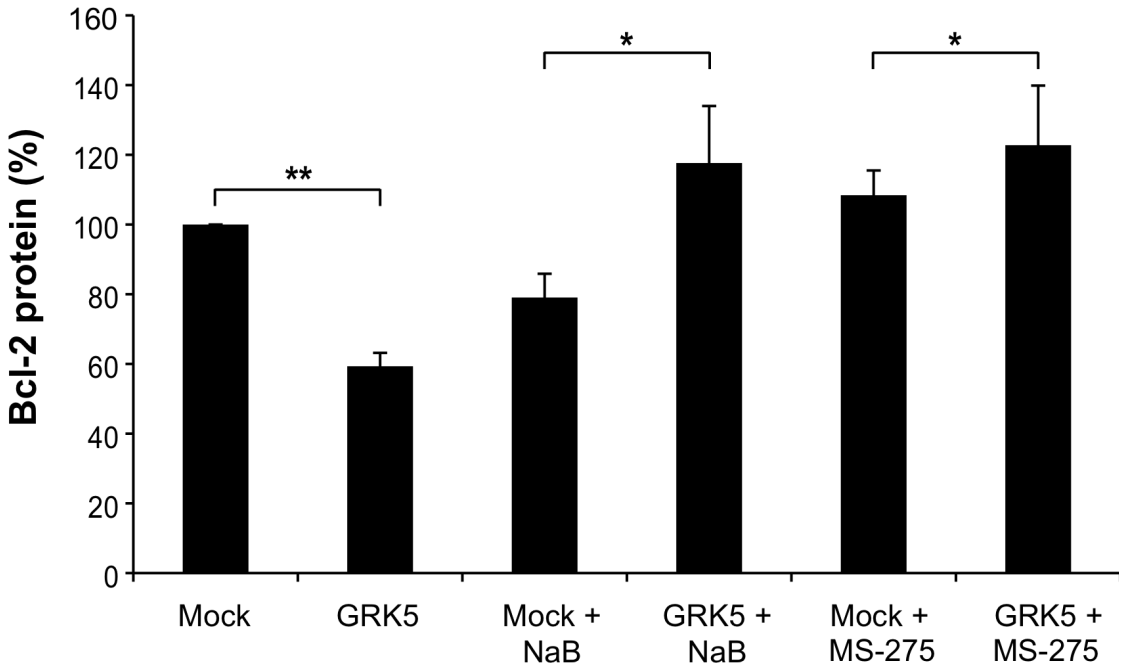


Figure 5.3 GRK5 represses Bcl-2 in an HDAC dependent manner

HT-29 cells were transfected as indicated and treated with DMSO (mock), sodium butyrate (NaB, 10 mM) or the class I specific HDAC inhibitor, MS-275 (10 μM) for 24 hours, and Bcl-2 protein expression detected by Western blot. The bar graph represents average Bcl-2 protein expression levels detected by Western blot, quantified relative to ERK expression levels, as a loading control and normalised to mock. Error bars represent standard error of the mean from three independent experiments. **P<0.05; *P<0.5.

5.3 GRK5 represses transcription

Chapter 3 identified class I HDACs and Sin3A as novel GRK5 binding partners. Moreover, data suggesting that GRK5 represses transcription of Bcl-2 in SHSY5Y cells has previously been reported [80]. In order to test whether GRK5 can act as a transcriptional regulator in HT-29 cells and whether the interaction of GRK5 with HDAC1 and Sin3A is required for transcriptional repression, I used a modified GRK5 construct in a pre-existing Gal4 luciferase assay. This assay utilises a firefly

luciferase reporter gene that is under the control of the Gal4 transcription factor. I compared firefly luminescence when the reporter gene was co-transfected with a Gal4 DNA binding domain (Gal4-DBD) that activates transcription, to that obtained when a Gal4-DBD-GRK5 (GRK5) fusion protein was expressed. A *Renilla* luciferase under the control of a constitutively active, cytomegalovirus (CMV) promoter, was also co-transfected in each condition as a measure of transfection efficiency. Figure 5.4 shows the average luminescence normalised relative to mock-transfected cells. GRK5 represses Gal4 mediated transcription, as the average luminescence fell from 452.8% ± 66.8 when Gal4-DBD was expressed, to 166.3% ± 18.7 when Gal4-DBD-GRK5 was expressed.

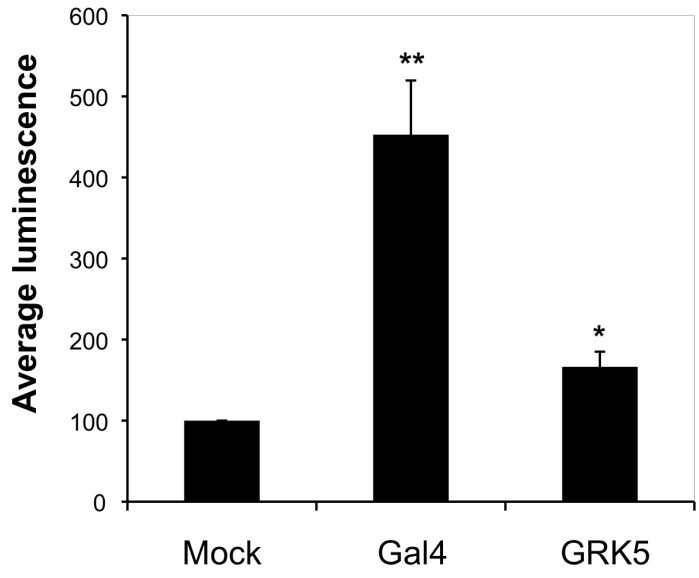


Figure 5.4. GRK5 represses Gal4 mediated transcription

HT-29 cells were transfected with either a mock, Gal4-DBD (Gal4) or Gal4-DBD-GRK5 (GRK5) construct. Cells were co-transfected with a firefly luciferase expression plasmid for Gal4 and a constitutively expressed *Renilla* luciferase plasmid and subjected to the luciferase assay 24 hours post transfection. Average luminescence is normalised to mock transfected cells. Error

bars represent standard error of the mean from three separate experiments; *P<0.05; **P<0.005 relative to mock.

In an attempt to determine whether the GRK5-mediated regulation of Gal4 controlled transcription is Sin3A dependent, I aimed to simultaneously knockdown Sin3A and overexpress Gal4-DBD-GRK5 and study the corresponding effects on transcription. Knocking down Sin3A by transfecting as well as nucleofecting Sin3A siRNA did not work, such that an alternative approach was used based on the competitive inhibition of the GRK5/Sin3A interaction. I co-expressed Gal4-DBD-GRK5 and the luciferase constructs with Myc-tagged mouse C-terminal Sin3A deletion mutants, as shown in Figure 5.5, and measured Gal4 mediated transcription 24 hours later. I hypothesised that interacting with overexpressed Sin3A may prevent Gal4-DBD-GRK5 from interacting with the endogenous Sin3A repressor complex and would thus prevent GRK5-dependent repression of the Gal4 luciferase target gene if this was a Sin3A-dependent event. A similar experiment has been performed to test the functional role of the interaction between Sin3A and the co-repressor, Alien. Transfection of Sin3A deletion fragments 545–678 (HID) and 1001–1337, both of which were previously shown to bind Alien directly, inhibited the Alien-mediated luciferase transcriptional repression by up to approximately 33% [178].

Figure 5.5A illustrates the Myc-tagged Sin3A deletion mutants used in the luciferase assay compared to the GST-tagged deletion fragments used in chapter 3 in direct binding assays. The C-terminal GST-Sin3A mutant encompassing residues 545–1157 (GST-545-1157) binds to GRK5 directly (Figure 3.6) and is highlighted in grey. The direct binding of GRK5 exclusively to this mutant suggests

that the interaction occurs somewhere between the HID and C-terminus of Sin3A. GRK5 would thus be predicted to bind to Sin3A N1015 and presumably Sin3A N680 if the HID represents the GRK5 binding site. Data in Figure 5.5B suggests that the GRK5-mediated repression of Gal4-mediated transcription is slightly but significantly relieved by these Sin3A fragments. Co-transfection of Gal4DBD-GRK5 with either N205 or N479 does not affect GRK5-mediated transcriptional repression, which is not surprising given that these fragments do not interact with GRK5 (Figure 5.5B).

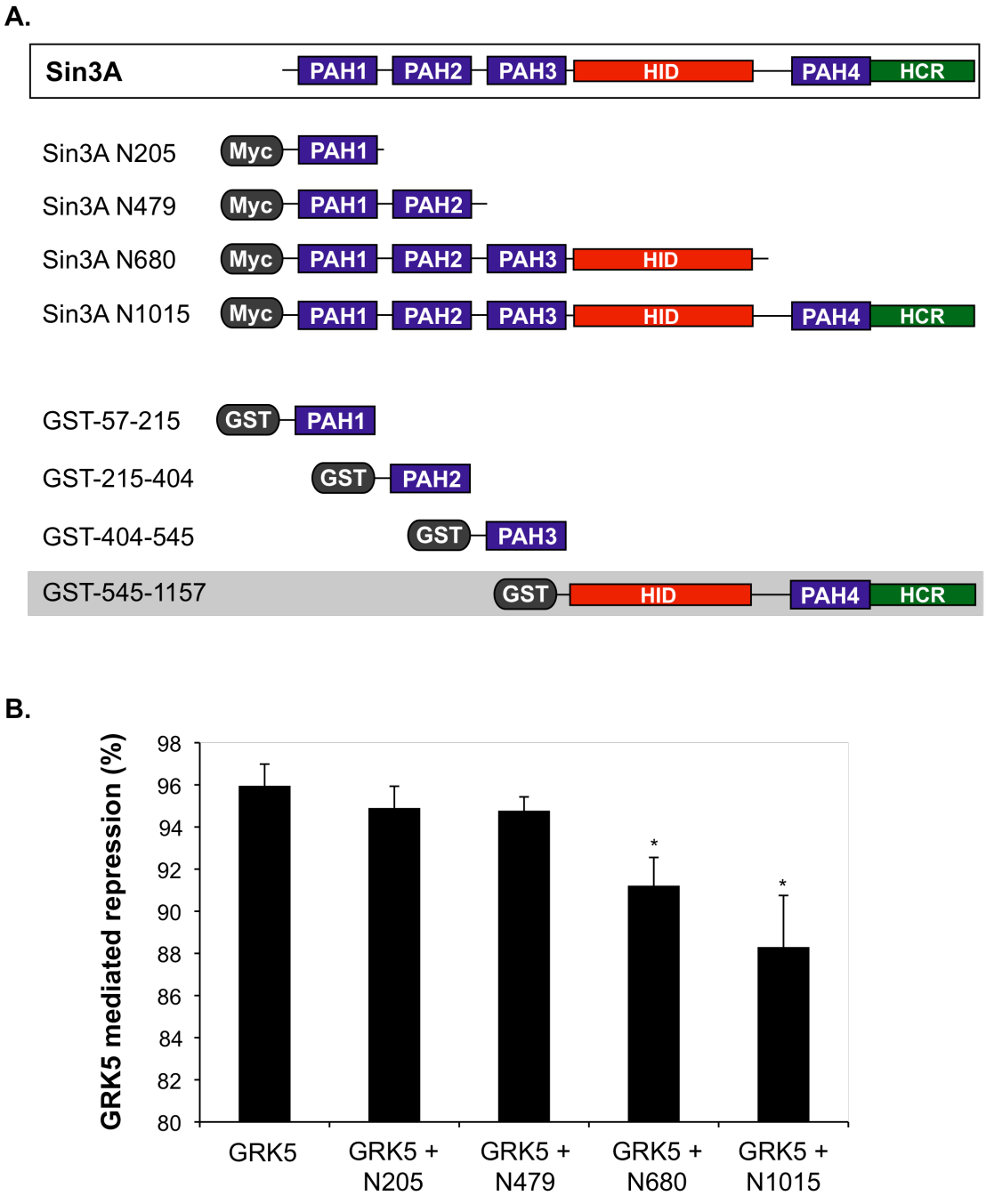


Figure 5.5 GRK5-mediated Gal4 transcriptional repression is Sin3A dependent

HT-29 cells were transfected with either Gal4-DBD (Gal4) or wildtype GRK5 fused to the Gal4-DBD (GRK5) and the mouse Myc-tagged Sin3A deletion mutant indicated (N205, N479, N680 or N1015). Cells were additionally co-transfected with a firefly luciferase expression plasmid for Gal4 and a constitutively expressed *Renilla* luciferase plasmid and subjected to the luciferase assay 24 hours post transfection. **A.** The schematic shows full length Sin3A and Sin3A deletion

mutants and the domains they encompass. The Myc tagged mutants used in the Gal4 luciferase assay are shown in comparison to the GST-tagged mutants used in chapter 3, with the mutant shown to interact directly with GRK5 highlighted in grey. **B.** The bar graph shows the percentage of GRK5-mediated repression of the luciferase reporter gene, calculated as the percentage reduction in luminescence relative to the luminescence reading following the overexpression of the Gal4 construct. Error bars are standard error of the mean from three separate experiments; *P<0.05 relative to GRK5.

Data from the two previous figures highlights a potential role for GRK5 in regulating transcription in a Sin3A-dependent manner. The Gal4 assay, however, is an artificial model of transcription and the possibility exists that the Gal4-DBD-GRK5 fusion protein may be inhibiting luciferase expression indirectly by, for example, sterically inhibiting the Gal4-DBD from interacting with the promoter. In order to ascertain directly whether GRK5 is capable of regulating transcription, I performed RT-PCR experiments to quantify mRNA levels of both GRK5 and Bcl-2, following transfection of GRK5 siRNA or control siRNA into HeLa cells. HeLa cells rather than HT-29 cells were used for these experiments, since HT-29 cells have extremely low levels of GRK5, whereas levels of the kinase are almost 7-fold higher in HeLa cells (Figure 5.1). Detecting GRK5 mRNA in HT-29 cells and, furthermore, detecting a reduction in mRNA levels following siRNA transfection, proved technically difficult. Figure 5.6 shows that knocking down GRK5 by 93% ± 0.01 causes a simultaneous increase in Bcl-2 mRNA levels of 22% ± 0.1. These data are consistent with findings in SHSY5Y cells, where GRK5 knockdown by approximately 70% caused a slight but significant increase in Bcl-2 mRNA by approximately 10%.

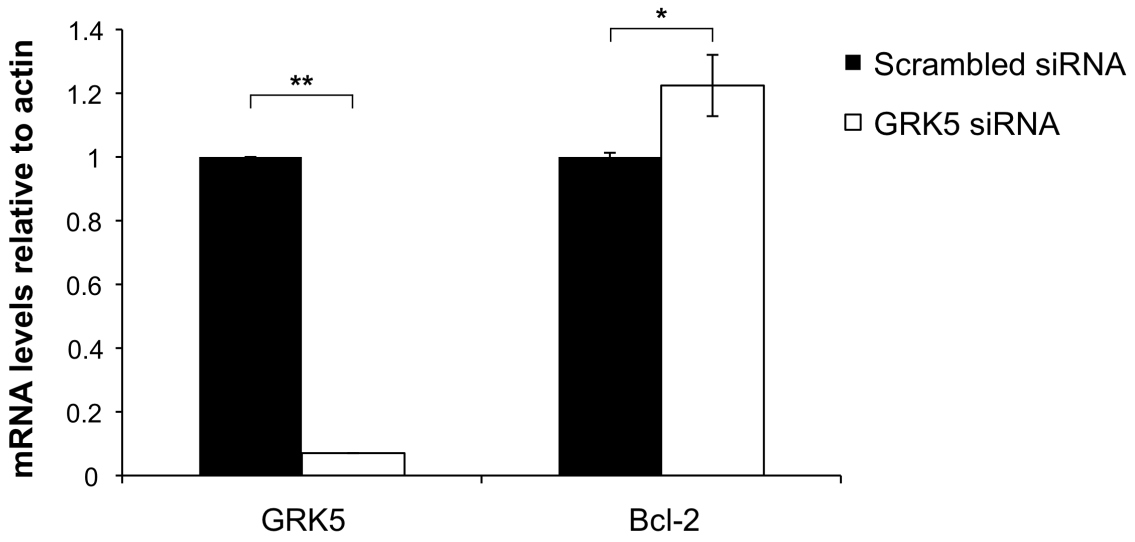


Figure 5.6 GRK5 represses Bcl-2 transcription

HeLa cells were transfected with GRK5 siRNA or scrambled siRNA and left 48–72 hours before RNA was extracted and purified. mRNA levels of GRK5 and Bcl-2 were quantified by RT-PCR relative to β-actin. This bar graph shows mean GRK5 and Bcl-2 mRNA levels relative to β-actin normalised to scrambled siRNA in each case. Error bars represent standard error of the mean from three separate experiments; *P<0.5, **P<0.05.

To summarise this section, GRK5-mediated down-regulation of Bcl-2 protein levels is sensitive to class I HDAC inhibitors, suggesting regulation is class I HDAC dependent. A Gal4-DBD-GRK5 construct inhibits Gal4 mediated transcription, which is modestly relieved by co-transfecting Sin3A mutants that are likely to bind to the kinase, implying that GRK5 represses transcription *in vitro* in a Sin3A-dependent manner. Furthermore, GRK5 negatively regulates Bcl-2 transcription in HeLa cells. Taken as a whole, these data suggest a role for GRK5 in regulating transcription, potentially in a HDAC and Sin3A dependent manner.

5.4 GRK5 increases apoptosis sensitivity

As previously explained, levels of the anti-apoptotic protein, Bcl-2, are upregulated in many cancers, including colorectal. Bcl-2 overexpression has also been related to chemotherapy resistance. Sections 5.1–5.3 show GRK5 to negatively regulate Bcl-2 protein and mRNA levels in cancerous cell lines. Considering the crucial role Bcl-2 plays in regulating apoptosis and how these mechanisms are altered in cancerous tissues, I wanted to investigate whether GRK5 is capable of influencing apoptosis in HT-29 cells. Figure 5.1 shows GRK5 levels to vary across a range of cancer cell lines, with OVCAR3 and HT-29 cells having the highest and lowest kinase levels respectively. Considering endogenous levels of GRK5 differ so greatly in these two cell lines, I wanted to ascertain whether this difference is reflected in different sensitivities to treatment with apoptosis-promoting agents. I treated both cell lines with increasing amounts of DOXO, a chemotherapeutic agent commonly used in many cancer treatment regimes, including that of ovarian cancer patients. Cells were harvested 24 hours post treatment and expression levels of cleaved-poly (ADP-ribose) polymerase-1 (PARP-1) detected by Western blot, as a measure of apoptosis. The induction of apoptosis results in the activation of caspases, which go on to degrade various cellular protein targets. The first of such caspase targets to be described was the nuclear protein, PARP-1, which is cleaved from its 116kDa form to fragments of 89 and 24kDa [179]. PARP-1 catalyses the synthesis of (ADP)-ribose polymers and this post-translational modification plays a key role in DNA repair and defence mechanisms against DNA damage [180, 181]. Caspase-mediated cell death is achieved by the cleavage of proteins like PARP-1, which are required for cell survival. The appearance of cleaved PARP-1 fragments thereby serves as a biomarker for apoptosis.

An initial experiment shows that increasing the concentration of DOXO results in increased levels of cleaved PARP-1 in both OVCAR3 and HT-29 cells, as indicated by Western blotting using an antibody specific to the 89kDa PARP-1 fragment (Figure 5.7). Interestingly, there is a positive correlation between endogenous GRK5 levels and the amount of cleaved PARP-1. OVCAR3 cells, which have approximately 10-fold more endogenous GRK5 (Figure 5.1) also have considerably more cleaved PARP-1 following DOXO treatment, compared to HT-29 cells (Figure 5.7). Considering GRK5 expression alone has little effect on cleaved PARP-1 levels, these data suggest that GRK5 may therefore be playing a role in regulating apoptosis in response to DOXO treatment.

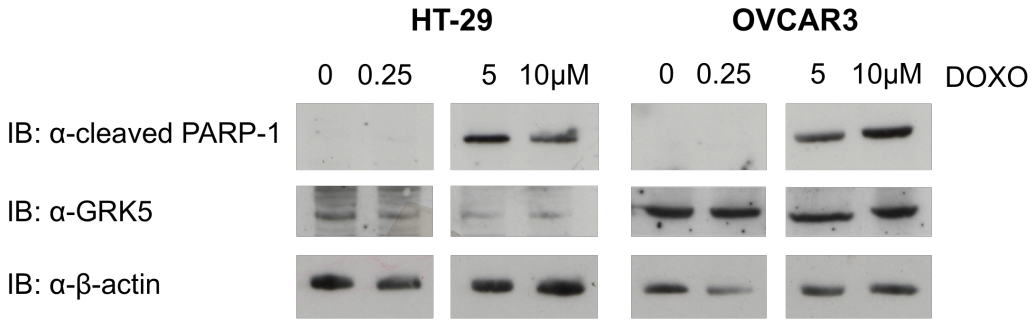


Figure 5.7 OVCAR3 cells express more cleaved PARP-1 following DOXO treatment compared to HT-29 cells

OVCAR3 and HT-29 cells were treated with increasing amounts of doxorubicin (DOXO) as indicated. Cells were harvested 24 hours later and lysates immunoblotted (IB) with an antibody against the 89kDa cleaved PARP-1 fragment. N = 1.

DOXO treatment is commonly given to ovarian cancer patients, whereas 5-FU is the backbone drug used to treat colon cancer. In order to further investigate whether GRK5 may enhance apoptosis sensitivity in colon cancer, I first wanted to test whether GRK5 could sensitise HT-29 cells to 5-FU treatment. I overexpressed GRK5, as indicated in Figure 5.8, and treated cells 24 hours later with 200 μM 5-FU. Apoptosis was measured 24 hours post treatment by two different assays: cells were stained with the viability dye, 7-aminoactinomycin D (7-AAD) and counted by fluorescence-activated cell sorting (FACS) (Figure 5.8A), and an immunofluorescence apoptosis assay was also performed, staining for cleaved caspase 3 expression levels (Figure 5.8B).

HT-29 cells were either mock transfected using pcDNA3 or transfected with increasing amounts of GRK5. Cells were treated with 5-FU or DMSO 24 hours post transfection and assayed 24 hours later. Cells were pelleted and incubated with 7-AAD, which inserts itself between successive cytosine/guanine bases, thus staining the DNA of apoptotic cells, as the chromatin is accessible. In an initial experiment, 10,000 cells were counted by flow-cytometry, with the percentage of cells stained with 7-AAD giving an indication of cell death. As can be seen in Figure 5.8A, GRK5 overexpression has some effect on DMSO treated cells, with cell death increasing from 9.4% in untransfected cells, to 19.5% when 7 μg GRK5 is transfected. The combination of GRK5 overexpression and treatment with 5-FU, however, causes an increase in cell death from 4.9% in untransfected cells to 30.1% when 7 μg GRK5 is transfected. Although this assay looked promising and suggested that GRK5 expression sensitises cells to 5-FU-induced apoptosis, transfection efficiency was only approximately 30% (data not shown). I thus sought an assay

that specifically looked at GRK5-transfected cells. While it is possible to transfect cells with a GFP-tagged GRK5 construct and measure percentage cell death in the population of GRK5-transfected cells by FACS, using a tagged construct is not ideal. Adding a tag, such as GFP on to a construct can distort its usual conformation, which may affect its cellular localisation and interaction with target proteins by masking protein binding sites. Nevertheless I did attempt to sort for GFP-GRK5 transfected cells but due to technical difficulties with the FACS machine it was not possible to obtain data. I therefore wanted to use a wildtype GRK5 construct to ascertain whether GRK5 has an effect on apoptosis. I decided to try an immunofluorescence based experiment, which is not only technically easier but would also enable the analysis of apoptosis specifically in GRK5-transfected cells.

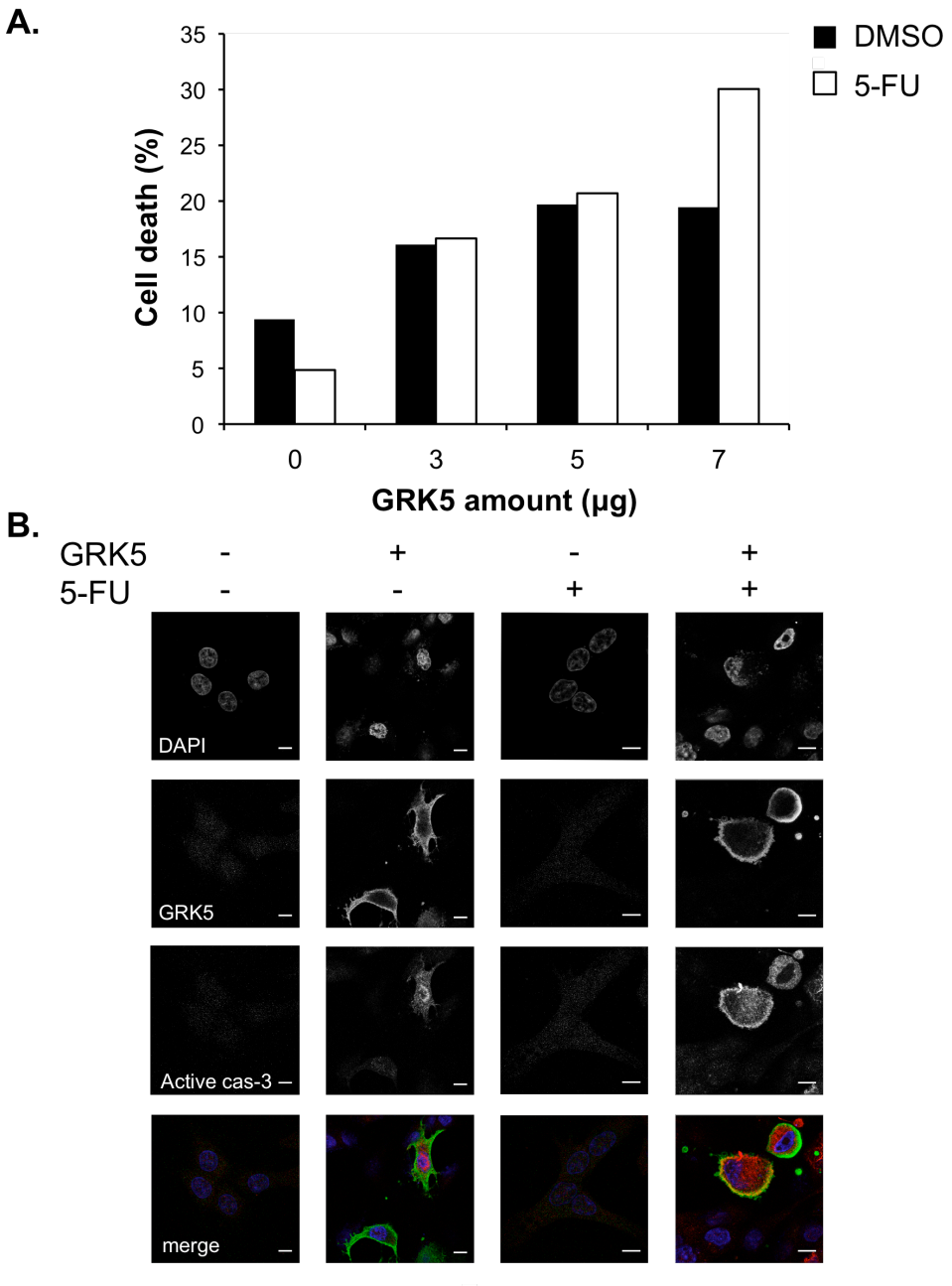

A.
DMSO
5-FU
Cell death (%)
35
30
25
20
15
10
5
0
0
3
5
7
GRK5 amount (µg)
B.
GRK5 - + - +
5-FU - - + +
DAPI
GRK5
Active cas-3
merge
C.
Mock
GRK5
*
Active cas 3 positive / total stained cells (%)
30
25
20
15
10
5
0
DMSO
5-FU

Figure 5.8 GRK5 enhances 5-FU induced apoptosis

HT-29 cells were transfected as indicated and treated or not with 5-fluorouracil (5-FU, 200 μM) 24 hours post transfection. **A.** Increasing amounts of GRK5 were transfected into HT-29 cells and apoptotic cells were sorted 48 hours later by flow cytometry using the 7-AAD viability dye. The percentage of cells positively stained with 7-AAD is shown relative to total cell number. **B.** Representative confocal images from three separate experiments following transfection with 2.5 μg GRK5 and treatment with 200 μM 5-FU. Cells were stained with an anti-active caspase 3 antibody (red), an anti GRK4-6 antibody (green) and Hoechst stain (blue). **C**. Quantification of cleaved caspase 3 staining by immunofluorescence. The number of cleaved caspase 3 cells were counted relative to the number of GRK5-transfected cells and expressed as a percentage of cell death. In mock-transfected conditions, nuclei were counted at random and then assessed for cleaved caspase 3 staining. Error bars represent standard error of the mean from three separate experiments; *P = 0.01.

HT-29 cells were grown on coverslips and transfected or not with GRK5, using the pcDNA3 vector as a mock, and treated with DMSO or 200 μM 5-FU 24 hours later. Cells were then stained using an anti-GRK4-6 antibody (green) to detect transfected cells and an active caspase 3 antibody (red) as an indicator of apoptosis (Figure 5.8B). Caspase 3, an effector caspase, is one of the later caspases that is cleaved and subsequently activated during the apoptosis caspase cascade, as explained in section 1.3.3.2. Caspase 3 is present as an inactive zymogen whose cleavage, which occurs following the induction of apoptosis, results in its activation. Caspase 3 is cleaved by caspase 8, 9 or 10 and goes on to activate caspase 6 and caspase 7. Detection of cleaved caspase 3, like cleaved PARP-1, thereby serves as an indicator of apoptosis. The number of GRK5-positive cells that were also positive for active caspase 3 were recorded as a measure of apoptosis. Under mock-transfected conditions, nuclei were counted at

random following DNA staining with the bisBenzimide Hoechst stain and subsequently assessed for cleaved caspase 3 staining to determine percentage cell death. Treatment with 5-FU does not significantly increase apoptosis in mock-transfected cells, as shown in the immunofluorescence images of Figure 5.8B, as well as in data quantified in Figure 5.8C; 0.77% ± 0.1 death occurred in DMSO-treated, mock-transfected cells, which increased to 0.93% ± 0.2 following 5-FU treatment. The overexpression of GRK5, however, greatly increased apoptosis, as can be seen from the positive caspase 3 staining in Figure 5.8B, as well as from data in Figure 5.8C. DMSO treatment caused 9.0% ± 0.5 of GRK5-overexpressing cells to undergo apoptosis, which increased to 22.4% ± 3.6 ($P = 0.01$) following 5-FU treatment. HT-29 therefore appear to be resistant to 5-FU treatment and GRK5 increases HT-29 cell death independently of 5-FU, as well as by enhancing treatment effects. Additionally, this assay also enabled monitoring of the cellular morphological changes in response to apoptosis. Cells positive for active caspase 3, following combined GRK5 overexpression and 5-FU treatment, appear more spherical in shape compared to untreated cells, which is indicative of apoptosis induction (Figure 5.8B).

In order to ascertain whether the nuclear localisation of GRK5 is necessary for GRK5-mediated apoptosis, I overexpressed the GRK5ΔNLS mutant, which is excluded from the nucleus, in HT-29 cells. Following 5-FU treatment the percentage of apoptotic cells overexpressing wildtype GRK5 was compared to cells overexpressing the GRK5ΔNLS mutant. Figure 5.9 shows that while GRK5 expression sensitises cells to 5-FU treatment, expression of the GRK5ΔNLS has a less pronounced effect. The percentage of caspase 3 positive cells increases from 2.1% ± 0.2 in mock-transfected cells to 20.0% ± 0.7 in GRK5-expressing cells. In

contrast, only 6.5% ± 0.9 of GRK5ΔNLS-expressing cells are caspase 3 positive after treatment with 200 μM 5-FU for 24 hours. The nuclear localisation of GRK5 is therefore important for its role in regulating apoptosis in HT-29 cells.

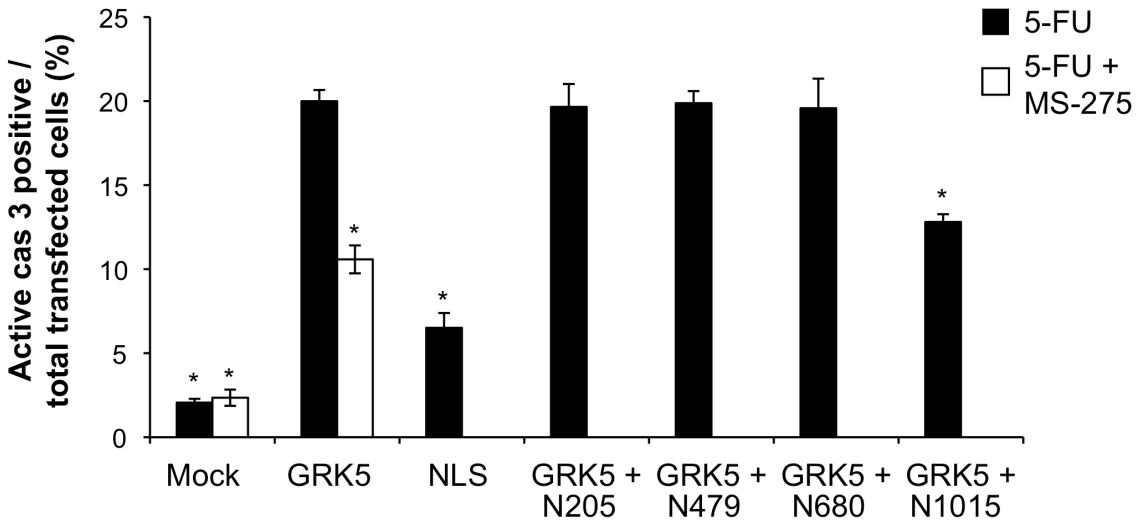


Figure 5.9 GRK5 enhances apoptosis in a class I HDAC and Sin3A dependent manner

HT-29 cells were transfected as indicated, with either a mock, GRK5 wildtype (GRK5), GRK5ΔNLS mutant (NLS), or wildtype GRK5 co-transfected with a Myc-tagged Sin3A mutant (N205, N479, N680 or N1015). At the time of transfection cells were treated with MS-275 (10 μM) as indicated. 24 hours post transfection, cells were treated with 5-FU (200 μM) and left for a further 24 hours. Cells were stained with an anti-GRK5 antibody (Mock, GRK5, NLS) or an anti-Myc antibody (GRK5 + N205, GRK5 + N479, GRK5 + N680, GRK5 + N1015), as well as an anti-cleaved caspase 3 antibody, as a measure of apoptosis. The percentage of transfected cells stained with cleaved caspase 3 were counted relative to total cell number, giving an indication of cell death. The bar graph shows mean data from at least 3 experiments. Error bars represent standard error of the mean. *P<0.001 relative to GRK5 5-FU treated cells.

To further probe the mechanism by which GRK5 is controlling apoptosis, cells were treated with 10 μM of the class I HDAC inhibitor, MS-275, 24 hours prior to 5-FU

treatment. Treatment with MS-275 does not affect apoptosis in mock transfect cells, as the percentage of caspase 3 positive cells is 2.1% ± 0.2 and 2.4% ± 0.5 in 5-FU treated and combined 5-FU and MS-275 treated cells respectively. MS-275 treatment does however reduce apoptosis in GRK5-transfected cells, as the percentage caspase 3 positive cells falls from 20.0% ± 0.7 following 5-FU treatment to 10.6% ± 0.8 following combined 5-FU and MS-275 treatment. GRK5 mediated apoptosis regulation is therefore class I HDAC dependent.

In order to determine whether Sin3A is equally important in GRK5-mediated apoptosis regulation, the Myc-tagged Sin3A C-terminal deletion mutants, as described in section 5.3, were co-transfected with GRK5. Cells were stained with an anti-Myc antibody and cleaved caspase 3 staining recorded in cells staining positive for Sin3A deletion mutants and GRK5. Co-expression of the longest Sin3A mutant, N1015, with GRK5 reduces apoptosis by 12.8% ± 0.4, compared to 20.0% ± 0.8 when GRK5 is expressed alone. Specific to the Sin3A N1015 mutant is the inclusion of PAH4 and the HCR in the construct, compared to the N205, N479 and N680 mutants, which only encompass PAH1, PAH1 and PAH2, and PAH1–3 and the HID, respectively (Figure 5.5A). In addition to inhibiting GRK5-mediated apoptosis, the N1015 mutant is also the most effective inhibitor of GRK5-mediated transcriptional repression of luciferase genes, as shown in Figure 5.5B. Data in Figure 3.6 suggests that GRK5 binds directly to a GST-Sin3A-545-1157 mutant that encompasses the HID, PAH4 and the HCR. The N1015 mutant, whose residues overlap with those included in the GST-Sin3A-5454-1157 mutant, is therefore likely to act as a Sin3A inhibitor, binding to GRK5 and preventing its interaction with the endogenous Sin3A repressor complex and thereby reducing

GRK5-mediated apoptosis. The N-terminal fragments, N205 and N479 do not bind to GRK5 and thus would not be predicted to inhibit the GRK5/Sin3A interaction. These mutants neither affect GRK5-mediated transcriptional repression nor apoptosis (Figure 5.5B and 5.9). The N680 mutant encompasses the HID, to which GRK5 may interact, and this mutant slightly reduces GRK5-mediated transcriptional repression (Figure 5.5) but does not affect GRK5-mediated apoptosis, as 19.6% ± 1.8 cells stain positive for caspase 3 compared to 20.0% ± 0.7 when GRK5 is transfected alone (Figure 5.9), suggesting the longest Sin3A construct binds most tightly to GRK5. These data imply that GRK5 increases apoptosis sensitivity in HT-29 cells following 5-FU treatment, in both a class I HDAC and possibly a Sin3A-dependent manner.

5.5 Inhibiting the micro RNA, miR-135, upregulates GRK5 and increases apoptosis sensitivity

Micro RNAs (miRNAs) are short non-coding RNA sequences of 21–23 nucleotides that bind target mRNAs, cause their degradation and thus repress gene expression at the level of translation. MiRNAs bind Argonaute proteins in the RNA-induced silencing complex, thereby directing the complex to specific complementary mRNA targets to mediate their degradation by the activation of RNAase in a process known as RNA interference [182]. The human genome encodes over 1000 miRNAs [miRBase 19, August 201], which are thought to regulate approximately one third of all human genes [183]. Considering the direct involvement of miRNAs in regulating gene translation, it isn't surprising that they have been implicated in tumourigenesis and cancer metastasis. MiRNA signatures are being investigated as diagnostic markers, as levels differ between benign and malignant tissues and even correlate

with differentiation states of cancers [184]. The upregulation of miRNA-135a and miRNA-135b are likely to be one of the earliest events in the progression of normal colorectal epithelium to early adenomas and represent a novel mechanism of APC and thus WNT signalling regulation; their upregulation correlates with reduced APC levels and both miRNAs reduce APC translation *in vitro* [185]. The inactivation of APC is a major initiating event in colorectal cancer development, occurring in over 60% of cases [186]. Manipulating endogenous miRNA levels as a therapeutic approach is currently being researched and tested through the use of specific miRNA mimics and oligonucleotides to stimulate upregulation, and anti-sense oligonucleotides and synthetic analogues to reduce levels. The use of a miRNA-122 inhibitor is now in a human phase II hepatitis C virus clinical trial. The virus hijacks miRNA-122, which is thereby required for viral replication, such that a miRNA-122 inhibitor should halt viral replication and thus the progression of liver disease [182]. Furthermore, miRNA-122 is upregulated in high-grade prostate tumours, thus suggesting that the development of such a miRNA inhibitor could be used as a cancer therapeutic [187].

Both mature miRNA-135a and miRNA-135b are predicted to target GRK5 mRNA and levels of miRNA-135 and GRK5 mRNA are upregulated and downregulated in GBMs respectively [188]. The upregulation of miRNA-135a and b in CRC and in HT-29 cells [185] may therefore account for the low levels of GRK5, such that manipulating miRNA expression could provide a novel approach to regulating levels of the kinase. In comparison to HT-29 cells, HeLa cells have negligible levels of miRNA-135a and b [185], such that I used this cell line as a control, to study whether transfection of either a mature human miRNA-135a mimic or an anti-sense

oligonucleotide could affect GRK5 protein levels and furthermore, apoptosis sensitivity. I transfected either the human miRNA-135a mimic or anti-sense oligonucleotide into each cell line, along with dsRed fluorescent protein, as a measure of transfection efficiency, and 48 hours later GRK5 protein expression levels were measured by Western blot. Cells were also assayed for apoptosis by immunofluorescence staining with a cleaved caspase 3 antibody. As shown in Figure 5.10Ai and ii, overexpression of the miRNA-135a mimic reduces GRK5 protein expression levels in both HeLa and HT-29 cells, which corresponds with a reduction in cleaved caspase 3 staining compared to mock transfected cells (Figure 5.10B). The percentage of HeLa cells staining positive for cleaved caspase 3 decreases from 11.6% ± 0.8 in mock transfected cells to 5.8 ± 0.8 following overexpression of the miRNA-135a mimic, and from 8.7 ± 0.6 to 6.0 ± 0.7 in HT-29 cells (Figure 5.10B). Overexpression of the antisense oligonucleotide, which inhibits endogenous miRNA-135a, causes the reverse effect in HT-29 cells, upregulating GRK5 protein expression levels, as well as cleaved caspase 3 staining to 14.8% ± 1.3. By comparison, GRK5 expression levels in HeLa cells are not affected by overexpressing the antisense oligonucleotide, and cleaved caspase 3 levels are not significantly affected either, as was hypothesised (Figure 5.10A and B). These data suggest that a miR-135a mimic and inhibitor can manipulate endogenous GRK5 proteins levels in cells and also affect apoptosis. These data imply that use of a miR-135a inhibitor may be a potentially novel and effective therapeutic strategy in the treatment of colon cancer by not only upregulating levels of APC but also, potentially, GRK5.

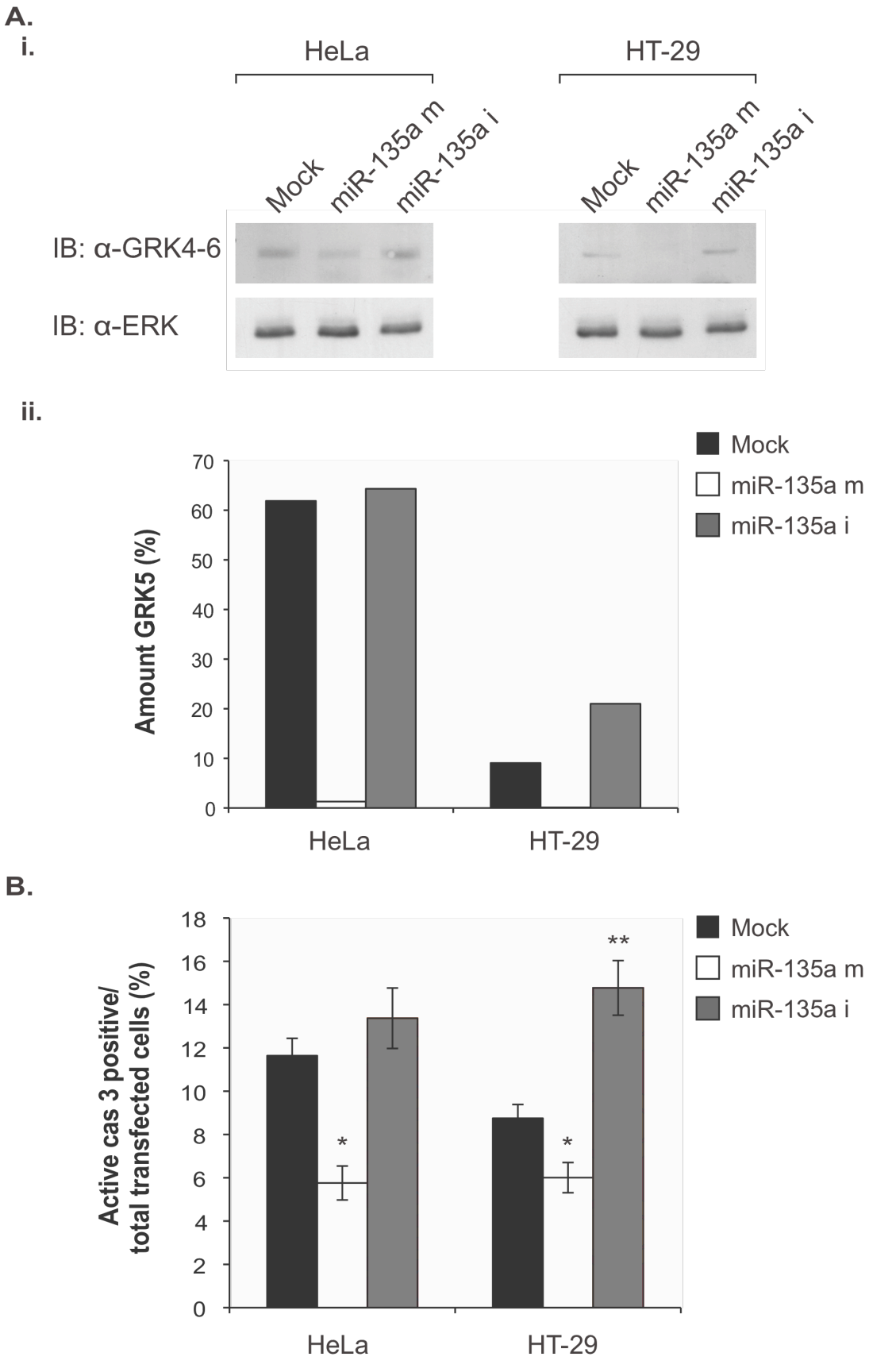

A.
i.
HeLa
HT-29
Mock
miR-135a m
miR-135a i
Mock
miR-135a m
miR-135a i
IB: α-GRK4-6
IB: α-ERK
ii.
Amount GRK5 (%)
70
60
50
40
30
20
10
0
HeLa
HT-29
Mock
miR-135a m
miR-135a i
B.
Active cas 3 positive/ total transfected cells (%)
18
16
14
12
10
8
6
4
2
0
*
*
**
HeLa
HT-29
Mock
miR-135a m
miR-135a i

Figure 5.10 A miRNA-135a mimic reduces GRK5 protein expression levels and apoptosis

HeLa and HT-29 cells were transfected with either a mock or 100 nM miRNA-135a mimic (m) or inhibitor (i) along with 10 nM dsRed for 48 hours. **A. i.** GRK5 protein expression levels were assessed by Western blot using an anti-GRK4-6 antibody. Total ERK levels were detected as a housekeeping gene using an anti-ERK antibody. ii. Quantification of A. The amount of GRK5 is calculated relative to the amount of ERK. **B.** Additionally, transfected cells were stained with an anti-cleaved caspase 3 antibody, as a measure of apoptosis. dsRed staining was used as a measure of transfection and the percentage of transfected cells stained with cleaved caspase 3 counted, giving an indication of cell death. The bar graph shows mean data from 3 experiments with error bars representing standard error of the mean. *P<0.05, **P<0.01 relative to mock transfected cells for each cell line. m = mimic; i = inhibitor

5.6 Summary

GRK5 binds to class I HDACs and Sin3A directly. The aim of this chapter was to investigate whether GRK5 could play a role in colon cancer pathogenesis by regulating transcription, in conjunction with HDACs and Sin3A. To summarise my findings:

- Grade II and metastatic colon cancer cells, HT-29 and SW620, respectively, have the lowest GRK5 levels in comparison to cervical, ovarian and prostate cancer cell lines.
- GRK5 overexpression reduces expression of the anti-apoptotic protein, Bcl-2, in HT-29 cells.
- The GRK5-mediated Bcl-2 repression is class I HDAC-dependent.
- GRK5 represses Gal4-mediated transcription in a Sin3A-dependent manner.
- GRK5 knockdown increases Bcl-2 mRNA levels, thus suggesting the negative regulation of Bcl-2 by GRK5 is at the transcriptional level.

- Compared to HT-29 cells, which have 10-fold less GRK5 protein, OVCAR3 undergo more apoptosis following DOXO treatment.
- GRK5 overexpression increases apoptosis in HT-29 cells following 5-FU treatment, as measured by FACS and immunofluorescence.
- GRK5 increases 5-FU induced apoptosis in HT-29 cells in a nuclear and class I HDAC dependent manner. Furthermore, overexpressing Sin3A mutant, N1015, which is likely to bind GRK5, significantly inhibits apoptosis, thus suggesting that GRK5-mediated apoptosis is also Sin3A dependent.
- Overexpression of a miR-135a mimic reduces GRK5 protein expression and apoptosis levels in HT-29 and HeLa cells.
- GRK5 protein expression levels and apoptosis are enhanced in HT-29 cells but not HeLa cells, following the overexpression of a miR-135a inhibitor.

Chapter 6. Discussion

This thesis describes the identification of novel GRK5 binding partners: class I HDACs, HDAC1, 3 and 8, and the repressor protein, Sin3A, and outlines a potential functional role for these interactions in inhibiting Bcl-2 transcription and increasing the sensitivity of the colon cancer cell line, HT-29, to chemotherapeutically induced apoptosis. Furthermore, GRK5 levels, which are downregulated in the diseased state, can be artificially upregulated by overexpressing a miR-135a inhibitor and this in turn increases apoptosis levels in HT-29 cells, thus highlighting a potential novel therapeutic strategy for the treatment of colon cancer.

GRK5 plays a causative role in pathological cardiac hypertrophy by acting as an HDAC5 kinase, thereby promoting the transcription of MEF2-regulated pro-hypertrophic genes indirectly, by relieving inhibition by HDAC5. [81]. The NLS and NTPB domains of GRK5, however, contain functional DNA binding sites, suggesting that GRK5 may additionally play a more direct role in controlling gene expression [11]. Class I HDACs function in a pro-hypertrophic manner in contrast to class II HDACs, which are anti-hypertrophic. Considering GRK5 and class I HDACs both play a causative role in pathological cardiac hypertrophy and that GRK5 can interact with DNA, the aim of my thesis was to investigate whether, in addition to its role as an HDAC5 kinase, GRK5 regulates HDAC activity and gene transcription directly, via a class I HDAC-dependent mechanism.

6.1 GRK5 as a transcriptional repressor

6.1.1 GRK5 binds class I HDACs and Sin3A

Chapter 3 identified class I HDACs and Sin3A as novel GRK5 interacting proteins. Relative to class II HDACs and HDAC2 binding, GRK5 binds strongly to the class I HDACs, HDAC1, HDAC3 and HDAC8 and these interactions are direct. The relative strength of the GRK5 interactions with class I HDACs compared to class II HDACs, as assessed by co-immunoprecipitation and pull-down assays, suggests that GRK5 may act as more than just a class I HDAC kinase. HDAC5 is a class II HDAC and a known GRK5 substrate. The relatively weak binding detected between these proteins may reflect the transient nature of the substrate/kinase relationship, which can be difficult to detect by co-immunoprecipitation [166]. In certain cases, binding of substrates and their corresponding kinases can only be detected using a kinase-dead mutant in co-immunoprecipitation experiments. Indeed, an interaction between the Ena/VASP like protein splice variant, EVL-1, and PKD was only detected in HEK293T cells following the overexpression of a kinase-dead PKD1 mutant [189]. Similar studies have been reported for the binding of thiamine pyrophosphokinase 1 to PKA substrates; binding to substrates was only observed with an inactive thiamine pyrophosphokinase 1 mutant, as opposed to the wildtype kinase [190].

Interestingly, the Koch group, when studying the substrate/kinase relationship of HDAC5 and GRK5, did manage to show by co-immunoprecipitation that GRK5 interacts with HDAC5 in whole cell lysates and nuclear extracts of NRVM [81]. The co-immunoprecipitation experiments performed investigated only a potential interaction between HDAC5 and GRK5, which was not compared to GRK5 binding

other HDACs or other known interactors. Figure 3.1B shows that GRK5 does interact with HDAC5 in HeLa cells, but that this interaction is less easily detected than the interactions with the class I HDACs, HDAC1, HDAC3 and HDAC8. My results do not contradict work by the Koch group, but rather emphasise the strength of the interactions between class I HDACs and GRK5, which may suggest an alternative role for GRK5 in regulating these HDACs in addition to, or instead of, its role as a kinase. GRK5 could, for example, be directly modulating the activity of class I HDACs or altering their cellular localisation. Work by the Qin group, who identified GRK5 as a negative regulator of Bcl-2 transcription in SHSY5Y cells, also found that knocking down GRK5 in this cell line significantly reduced HDAC activity by up to 70% [80]. I have shown GRK5 to negatively regulate Bcl-2 transcription in HT-29 cells and results from an *in vitro* Gal4 luciferase assay suggests that GRK5-mediated transcriptional repression is Sin3A dependent. Furthermore, I have shown that GRK5-mediated negative regulation of Bcl-2 protein expression levels is class I HDAC dependent. I would therefore predict that in HT-29 cells, like in SHSY5Y cells, GRK5 might be enhancing HDAC activity. While the Qin group did not show which class of HDAC GRK5 regulates, I hypothesise that GRK5 may be specifically upregulating class I HDAC activity. It would be interesting to test whether the catalytic activity of GRK5 is necessary for mediating not only the binding to class I HDACs and Sin3A, but also whether it is required for GRK5-mediated Bcl-2 transcriptional regulation and potentially class I HDAC activity, which is often upregulated by phosphorylation.

That GRK5 interacts relatively poorly with HDAC2 as compared to HDAC1, as shown by co-immunoprecipitation in HeLa cell lysates, by immunofluorescence in

HEp2 cells and by *in vitro* direct binding experiments, is somewhat surprising considering the high degree of sequence homology between HDAC1 and HDAC2. As discussed briefly in section 3.2, while HDAC1 and HDAC2 have some redundant and compensatory roles, several functions specific to each HDAC have also been identified. The global and targeted deletion of HDAC1 in mice results in early embryonic lethal death and a down-regulation of cellular HDAC activity is reported in HDAC1-null embryonic stem cells, despite the compensatory upregulation of HDAC2 [168]. HDAC1 and HDAC2 share 91% sequence identity at the amino acid level in the combined N-terminal and catalytic domains but are only 34% identical in their C-termini, thus suggesting a GRK5 binding site may be located in the C-terminus of HDAC1 [170]. The HDAC1 C-terminus is essential for its function, responsible for controlling its association with co-factors including Sin3A and its core component, RBBP4, as well the enzyme's catalytic activity. A C-terminal HDAC1 truncated mutant displays perturbations in both of these cellular phenotypes, as does the mutation of residues Ser421 and Ser423 to alanine, which are critical C-terminal HDAC1 phosphorylation sites [191]. It is therefore possible that phosphorylation of these residues by GRK5 or another kinase, such as casein kinase II, which phosphorylates these residues in T-Ag Jurkat cells [191], might be a prerequisite for GRK5 binding to HDAC1. HDAC2, by contrast, is phosphorylated at different C-terminal sites compared to HDAC1 [192], which may induce a conformational change to the protein that does not enable GRK5 binding. Performing co-immunoprecipitation experiments with wildtype GRK5 and a S421/423A HDAC1 mutant would confirm whether indeed C-terminal phosphorylation of HDAC1 is required for GRK5 binding and using a kinase dead

GRK5 mutant would ascertain whether the catalytic activity of GRK5 is necessary for mediating the binding event.

A serendipitous discovery from a mass spectrometry screen by the Ma group identified the mammalian transcriptional repressor protein, Sin3A, as a potential interactor of GRK5 [171]. Proteins in complex with GRK5 in MDA-MB-231 cells were analysed by mass spectrometry, following their separation on SDS-PAGE gels. Major GRK5-interacting proteins identified include DDB1, a subunit of the CUL4-ROC1 E3 ubiquitin ligase complex. DDB1 was further characterised by the group to be involved in regulating GRK5 levels; DDB1 binds and thus recruits GRK5 to the ubiquitin ligase complex, leading to its ubiquitination and degradation [171]. An additional hit from the screen that was not followed up by the group was the mammalian transcriptional repressor protein, Sin3A.

By co-immunoprecipitation experiments I have shown GRK5 interacts with Sin3A in HeLa cells. This interaction is direct and is mediated by the Sin3A C-terminal fragment, the region of the protein where components of the core complex assemble. The identification of Sin3A as a bona fide binding partner of GRK5 suggests a potential method whereby GRK5 could regulate HDAC function. HDACs are known to act in concert with accessory proteins, functioning as the enzymatic members of repressor complexes, which rely on recruiting transcription factors to target their activity to specific genes. Indeed, HDAC1 is a core component of the Sin3A complex and along with HDAC2 is responsible for the deacetylase activity of the repressor complex, which is required for full transcriptional repression by Sin3A [133]. The direct interaction of GRK5 with both HDAC1 and Sin3A is highly

suggestive of a functional role for the kinase in repressing transcription of Sin3A-regulated genes. *In vitro* direct binding assays detailed in chapter 3, show GRK5 binds directly to Sin3A between residues 545–1157. This region encompasses the HID, PAH4 and the HCR. The core Sin3A components, which include HDAC1, HDAC2, RBBP4, RBBP7, SAP18, SAP30 and SDS3, assemble on Sin3A in a region encompassing the PAH3–HID (Figure 6.1). The HID spans amino acids 545–678 and is the best characterised domain in the C-terminus of Sin3A. PAH4 and the HCR have few identified binding partners, namely O-GlcNAc transferase and the nuclear co-repressor, Alien, respectively [178, 193]. HDAC2 binds to the HID of Sin3A and this domain is necessary and sufficient to mediate transcriptional repression *in vitro*, implying that HDAC1 also binds this domain [161]. Neither HDAC1 nor HDAC2 have been shown to bind directly to Sin3A but instead the SDS3 protein, which also binds to the HID, bridges the gap between HDAC1/HDAC2 and Sin3A and augments HDAC activity [139, 148].

Considering GRK5 binds to HDAC1 and Sin3A directly it may also be involved in HDAC recruitment to Sin3A, possibly functioning as a ‘core’ Sin3A component. Including Sin3A there are eight core mammalian Sin3A complex proteins, which are highly conserved from yeast to man and were identified by affinity purification experiments. The mammalian Sin3A complex elutes over a broad range from size exclusion columns, suggesting many Sin3A complexes may exist within one cell. Results from multiple laboratories identified HDAC1, HDAC2, RBBP4, RBBP7, SDS3, SAP30 and SAP18 to associate with Sin3A with high stoichiometry and were thus labelled as ‘core’ Sin3A complex proteins (Figure 6.1). In the years following the identification of the core complex, a number of other Sin3A-associated proteins have been identified, including additional SAP proteins, SAP130, SAP25

and SAP180, which are known as accessory proteins and whose functions are largely unknown [139, 194]. The terminology ‘core’ or ‘accessory’ protein is somewhat ambiguous, possibly depending on the time at which the binding partner was identified, its conservation in yeast and whether a discernable function for its interaction with Sin3 has been found.

Purification of the Sin3A repressor complex in a gel filtration experiment and Western blotting, probing with anti-GRK5 antibodies, would confirm whether indeed GRK5 is a component of the repressor complex. Simultaneously probing for other Sin3A components would also ascertain whether GRK5 binding to Sin3A is in conjunction with the core complex, or whether the Sin3A/GRK5 interaction represents a distinct function of this master scaffold protein. GRK5 may specifically recruit HDAC1 to Sin3A or to SDS3 and through these interactions indirectly bind to HDAC2. It would be interesting to perform co-immunoprecipitation experiments to test whether indeed GRK5 can interact with SDS3 and whether GRK5 is important for HDAC binding to the repressor complex. Knocking down GRK5 in HeLa cells and then performing co-immunoprecipitation experiments to identify interactions between HDACs and Sin3A would identify whether GRK5 is important for these binding events. If GRK5 was found to be a crucial Sin3A binding partner required for complex formation and/or function, it may still be classed as an accessory, rather than a core complex protein. This would most likely be due to the delayed identification of GRK5 as a Sin3A binding partner, given that the core complex proteins were identified and labelled as such in the 1990s.

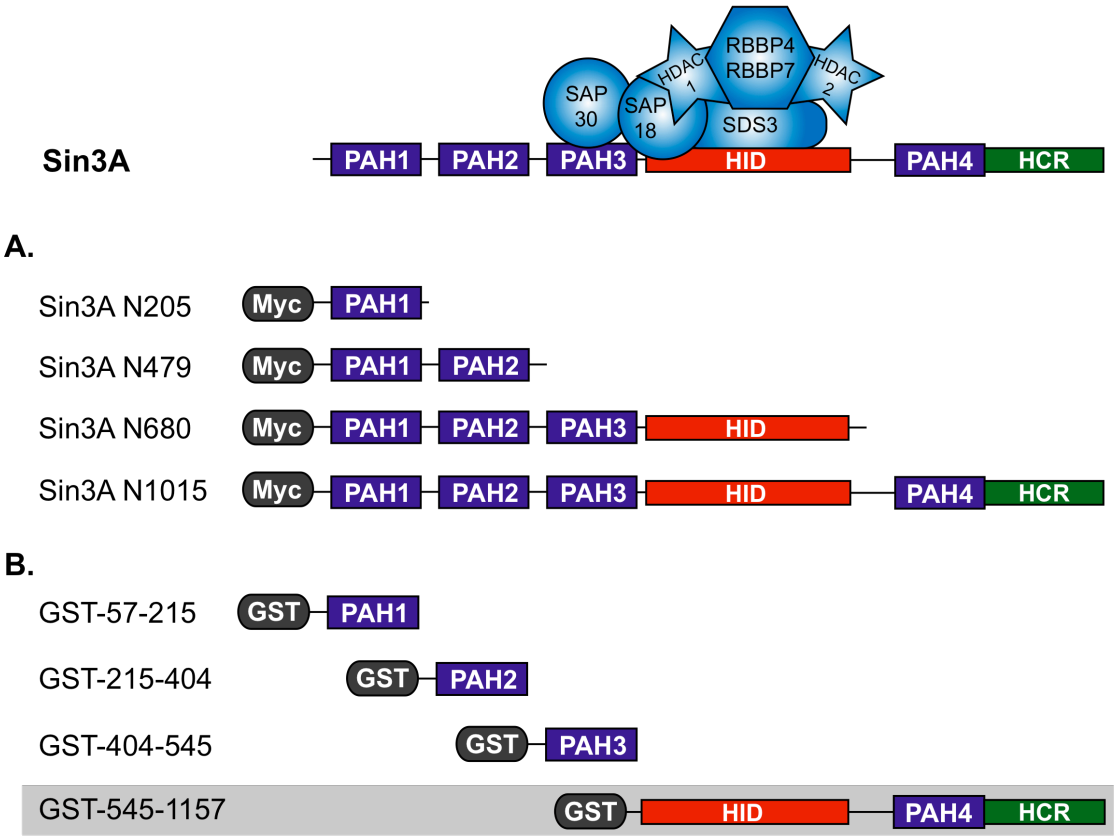


Figure 6.1 Full-length Sin3A and deletion mutants used in Chapter 5

A schematic representation of full length Sin3A is shown, with the core complex proteins in blue, assembling between PAH3–HID. **A.** A schematic representation of the Myc-tagged Sin3A mutants used in the Gal4 luciferase assay and the apoptosis assay. **B.** A schematic representation of the GST-tagged Sin3A mutants used in the direct binding assays are illustrated, with the longest mutant, which binds GRK5 directly, shaded in grey.

Additional binding partners of the Sin3A complex have also been identified, including the transcription factor, p53, and Rb, which are thought to bring specificity to Sin3A function by targeting the complex to specific genes. These binding partners were identified by co-immunoprecipitation experiments from cell lysates, much like the ones I performed to identify GRK5 as a Sin3A interacting protein. Following the identification of the protein-protein interactions, a potential functional role for the interaction was investigated. P53 binds to Sin3A between PAH2 and

PAH3 and this interaction is critical for p53-mediated gene repression, as Sin3A binding prevents p53 degradation [195, 196]. Rb recruits the Sin3 complex via Rb-binding protein 1 that binds directly to SAP30 and this interaction may account for the Rb-dependent transcriptional repression of E2F1-dependent promoters in quiescent cells [197]. Instead of acting as a core or accessory protein to the Sin3A complex, there is potential for GRK5 to act like a transcription factor and direct the Sin3A complex to the promoter of the Bcl-2 gene, rather than regulating the repressor complex assembly and function as a whole. That being said, most additional Sin3A binding partners interact with the repressor at PAH2 and data from the *in vitro* direct binding assay in chapter 3 identified a direct interaction of GRK5 with Sin3A in the C-terminus, between the HID, PAH4 and the HCR. I therefore propose GRK5 to play a role in regulating the Sin3A complex in a mechanism that may not only be limited to repression of the Bcl-2 gene.

6.1.2 GRK5 as a transcriptional repressor

Experiments in chapter 5 illustrated the potential capabilities of GRK5 to act as a transcriptional repressor. Using a Gal4 luciferase assay, which has been used previously to identify bona fide Sin3-interacting proteins that have a functional role in transcriptional repression, I was able to show that GRK5, when fused to the Gal4-DBD, represses transcription of Gal4 target genes *in vitro*. Furthermore, using Sin3A deletion mutants I showed that Gal4-mediated gene repression by GRK5 might be Sin3A dependent. GRK5 binds Sin3A between residues 545–1157, identified by *in vitro* direct binding assays using GST-Sin3A mutants, which are illustrated in Figure 6.1B, with the longest mutant that binds GRK5 directly, shaded in grey. Figure 6.1 compares the GST-Sin3A mutants used in the direct binding

assays shown in chapter 3, to the Myc-Sin3A mutants used in the Gal4 luciferase assay and in the apoptosis assays in chapter 5. The overexpression of GRK5 with Myc-tagged Sin3A mutants encompassing residues 1–680 (N680) or 1–1015 (N1015) (Figure 6.1A) modestly but significantly relieves GRK5-mediated luciferase gene repression, I predict, by competing with the endogenous Sin3A complex for GRK5 binding and thus preventing the GRK5/Sin3A complex localisation at the Gal4 promoter. In support of my hypothesis, the Myc-Sin3A deletion mutants that don't encompass residues 545–1157, N205 and N479, as illustrated in Figure 6.1, do not relieve GRK5-mediated transcriptional repression. Unlike GRK5, whose transfection efficiency was approximately 30%, the Sin3A deletion mutants were less well expressed in HT-29 cells. It is therefore likely that not all cells expressing the Gal4-DBD-GRK5 construct were also expressing the Sin3A mutant. The Gal4 luciferase assay does not discriminate between transfected and untransfected cells and the relatively low number of HT-29 cells co-expressing Gal4-DBD-GRK5 and Sin3A deletion mutants may therefore account for only a modest relief of GRK5-mediated transcriptional repression by N680 and N1015. Results from the Gal4 luciferase assay suggest that Sin3A residues 545–680, which encompass the HID, may be most critical for GRK5 binding. However, although not statistically different, the GRK5-mediated luciferase gene repression is relieved slightly more by co-expressing Gal4-DBD-GRK5 with N1015 compared to N680, which implies that the C-terminal Sin3A domains, PAH4 and the HCR, may provide additional stability to the GRK5/Sin3A interaction.

Considering GRK5 is capable of regulating transcription in an *in vitro* system, I then wanted to investigate whether the kinase could regulate transcription of the anti-

apoptotic protein, Bcl-2, in the colon cancer cell line, HT-29. GRK5 expression levels are predicted to be downregulated in high-risk patients and colon cancer cell lines and I observed that HT-29 and SW620 cells, both of which are derived from colorectal adenocarcinomas, have relatively low GRK5 levels in comparison to other tumour derived cell lines (Figure 5.1). Since Bcl-2 levels are upregulated in colon cancer and GRK5 negatively regulates Bcl-2 transcription in an unrelated cell line, SHSY5Y [80, 116], I investigated whether the low GRK5 expression levels were playing a causative role in colon cancer pathogenesis. Extending the original study by the Qin group, who observed a modest but significant increase in Bcl-2 mRNA levels of approximately 10% following GRK5 knockdown in SHSY5Y cells, I performed a knockdown of GRK5 in HeLa cells, which increased Bcl-2 mRNA levels by 22% ± 0.1. Like the Qin group, I also showed GRK5 to negatively regulate Bcl-2 protein expression, both by knocking down GRK5, as well as by overexpressing the kinase. I further showed the repression of GRK5-mediated Bcl-2 protein expression is dependent on class I HDACs, as treatment with the class I specific inhibitor, MS-275, abolished the reductions in Bcl-2 protein levels observed following overexpression of the kinase. While the Qin group did not use HDAC inhibitors, the concomitant upregulation of HDAC activity in SHSY5Y cells following GRK5 knockdown, supports their hypothesis that GRK5 negatively regulates Bcl-2 transcription in SHSY5Y cells by upregulating HDAC activity. They propose this mechanism may play a role in PD pathogenesis by regulating apoptosis, although they do not investigate this hypothesis [80]. My data supports and extends their work, confirming that indeed, GRK5 expression levels enhance the apoptotic sensitivity of HT-29 cells to the chemotherapeutic agent, 5-FU, but also independently of drug treatment. Moreover, I show that GRK5-dependent apoptosis

is Sin3A-dependent, thus providing a mechanistic explanation for the Qin group's findings, implicating the Sin3A complex and potentially HDAC activity in GRK5-mediated Bcl-2 gene repression.

The Qin group performed a chromatin immunoprecipitation (ChIP) experiment in SHSY5Y cells and showed GRK5 to interact with the Bcl-2 promoter. Using anti-GRK5 antibodies to immunoprecipitate GRK5 from cell lysates, the corresponding DNA bound to GRK5 was purified and detected by RT-PCR using primers specific for the Bcl-2 promoter. In control SHSY5Y cells, GRK5 bound to the Bcl-2 promoter but this interaction was significantly reduced in SHSY5Y cells overexpressing the GRK5 shRNA [80]. Given more time, I would have liked to perform a ChIP experiment in HT-29 cells to investigate GRK5 binding at the Bcl-2 promoter. Assuming that the results in SHSY5Y cells hold true in HT-29 cells, which is possible considering that GRK5 negatively regulates Bcl-2 transcription in both cell lines, it would also be interesting to test whether HDAC and Sin3A interactions are necessary to mediate the potential interactions of GRK5 with the Bcl-2 promoter. Given my data showing the importance of class I HDACs and the interaction with Sin3A in mediating Bcl-2 repression, I would predict GRK5 to bind to the Bcl-2 promoter in conjunction with the Sin3A complex. Sin3A binds to many promoters in differentiated myotubes to maintain muscle cell differentiation [198], and the complex also binds the promoter region of the transcription factor gene, *E2F1* and the prostate suppressor antigen gene and enhances gene repression by Ebp1 [199]. In some cases, however, the complex does not function as an independent transcriptional regulator and instead relies on the association of other proteins in order to repress transcription. In keratinocytes, for example, Sin3A binds to the

telomerase promoter, hTERT, in complex with the repressor protein, NFX1-91. Degradation of NFX1-91 dissociates the Sin3A complex from the promoter and initiates hTERT transcription [200]. Perhaps GRK5 is recruiting Sin3A to the Bcl-2 promoter. If indeed Sin3A binds to the Bcl-2 gene, it would be interesting to note whether this interaction is dependent on GRK5.

Both the Qin group and myself have highlighted the Bcl-2 gene as a cellular target of GRK5 transcriptional repression. Extending their work, my data in HT-29 cells thereby implicates GRK5 as a causative factor in colon cancer, as I have shown GRK5 protein levels are downregulated in colon cancer cells, HT-29 and SW620, and Bcl-2 upregulation is associated with the early development of CRCs [116]. While the Qin group showed GRK5 to negatively regulate Bcl-2 protein and mRNA levels in SHSY5Y cells, by Western blot and by RT-PCR respectively, following the overexpression of GRK5 siRNAs [80], my data gives mechanistic insight into this regulation, highlighting a possible role for GRK5 within a well-established repressor complex. An obvious question to pose is how GRK5 is acting, potentially in concert with Sin3A and HDAC1, to mediate gene repression. One possibility, as mentioned briefly in section 6.1.1 is by directing the complex to gene targets. Sin3A itself does not posses DNA binding capabilities, but depends on binding to transcription factors to target the HDAC activity of the complex to specific promoters. The majority of transcription factors that bind Sin3A do so via the N-terminal PAH1 and PAH2 domains and in fact no such interactions have been reported to occur at PAH3, PAH4 or the HCR. The nuclear co-repressor, Alien, however, binds Sin3A at the HID and HCR [139, 148]. Mammalian Sin3A was originally identified as a binding partner of the Mad family of repressor proteins and the formation of a

ternary complex of Sin3A with the Mad-Max heterodimer results in transcriptional repression of c-Myc. Mad1 binds to the PAH2 domain of Sin3A via a sequence of 13 amino acids in a conserved region of Mad1 that is known as a Sin3A interaction domain [201]. A PAH2-interacting consensus sequence has since been identified and is common to not only the Mad family but other transcription factors including transforming growth factor β inducible early response gene 2 (TIEG2) [202]. Transcription factors target Sin3A to specific genes and GRK5 may also play a role in this process. Perhaps GRK5, by virtue of its ability to bind Sin3A at sites distinct from other transcription factors, modifies the promoter-binding characteristics of specific transcription factor/Sin3A complexes.

The NTPB and NLS domains of GRK5 contain DNA binding sites. Based on the GRK6 homodimer crystal structure, the NLS and NTPB are oriented at opposite ends, such that a homodimer may wrap itself around DNA with the NLS and NTPB domains forming direct contacts with the nucleotides [11, 19]. As discussed in section 1.1.1, GRK6 was crystallised by the Tesmer group as a dimer, via a surface of its RH domain that is shared between the GRK4 and GRK1 subfamily members. The Tesmer group showed that dimerisation is not required for receptor phosphorylation, as GRK6 mutants with residues at the dimer interface mutated to alanine, phosphorylated rhodopsin to the same degree as wildtype GRK6 in COS-1 cells [19]. Lan Ma's group have shown that GRK5 can dimerise in HEK293 cells and they propose that dimerisation works to crosslink F-actin into bundles at the plasma membrane, a process which is responsible for neurite outgrowth and the formation of dendritic spines [25]. These data suggest that perhaps the conformation of the GRK5 dimer is important for binding macromolecules, such as

actin polymers and DNA. To investigate this hypothesis it would be interesting to test whether a C-terminal GRK5 mutant that fails to bind actin also fails to interact with DNA.

GRK5 may also be regulating Sin3A-mediated repression of gene transcription via its kinase activity. Phosphorylation is a known regulatory mechanism for abolishing interactions of Sin3A with transcription factors. Indeed, phosphorylation of TIEG2 at four sites near the Sin3A interaction domain disrupts binding to mouse Sin3A, resulting in loss of repression. Signalling via epidermal growth factor inhibits TIEG2 repression via a Ras/MEK1/ERK2 signalling pathway [202]. I have demonstrated that GRK5 overexpression in HT-29 cells increases Bcl-2 repression and that GRK5 knockdown causes the reverse effect. If GRK5 is playing a kinase-dependent role in regulating Sin3A it would be to enhance such interactions. Additionally, the enzymatic activity of HDAC1 is enhanced by phosphorylation [191]. In SHSY5Y cells, where GRK5 has been shown to negatively regulate Bcl-2 transcription, knockdown of GRK5 also reduced HDAC activity by up to 70% [80]. GRK5 may therefore be working to activate HDAC1 in the Sin3A complex to promote the repression of Bcl-2 gene transcription. Initial fluorometric HDAC activity assays were carried out to investigate whether GRK5 regulates HDAC activity in HT-29 cells, but due to technical difficulties clear results were not obtained. It would be worthwhile testing whether the GRK5ΔK215R kinase dead GRK5 mutant is capable of repressing Bcl-2 transcription, as well as interactions of GRK5 with class I HDACs and Sin3A, to shed further light on the mechanistic role played by GRK5 in the Sin3A repressor complex.

Many proteins involved in transcriptional repression bind to multiple repressor complexes, such as HDACs 1 and 2, which bind not only to Sin3A but also to NuRD, N-CoR, SMRT and CoREST. SAP30 is a core component specific to the Sin3A complex and while it is not responsible for the intrinsic repressor activity of the complex, it is involved in Sin3A-mediated N-CoR repression, by acting as a bridging factor between the two repressors [203]. GRK5 might play a similar role, recruiting accessory proteins to Sin3A, including other transcriptional repressors. N-CoR binds HDAC3 as part of its core complex. Data in Figure 3.2 shows GRK5 binds directly to HDAC3, albeit to a lesser extent than HDAC1 and HDAC8. There is, therefore, the potential for GRK5 to be involved in N-CoR-mediated repression, possibly by bridging the gap between N-CoR and Sin3A via its interaction with Sin3A and HDAC3. GRK5 could also potentially be recruited to N-CoR solely via HDAC3 binding. Whether GRK5 is involved in other mammalian repressor complexes, such as N-CoR, remains to be determined but could be tested by co-immunoprecipitation experiments using cell lysates. Considering Sin3A also binds to N-CoR, it would be interesting to investigate whether Sin3A would be necessary to mediate the interactions of GRK5 with not only N-CoR, but also other mammalian repressor complexes.

6.2 GRK5 is pro-apoptotic in cancer cells

Cancer, like many disease states, is associated with altered gene expression. Given that GRK5 binds to class I HDACs as well as the repressor protein, Sin3A, I wanted to investigate whether the kinase could play a role in colon cancer pathogenesis, potentially through modulating gene expression at the transcriptional level. Evidence in the literature suggests that levels of GRK5 are altered in colon

cancer, with high-risk patients showing reduced expression of the kinase and I have shown GRK5 protein levels are lowest in colon cancer cells in comparison to other tumour-derived cell lines. An obvious physiological consequence of repressing Bcl-2 levels is enhanced apoptosis. I compared apoptosis in OVCAR3 and HT-29 cells. OVCAR3 cells express approximately 10 times more GRK5/unit actin than HT-29 cells. At the highest level of GRK5 expression tested, 2.5-times as many OVCAR3 cells underwent apoptosis as compared to HT-29 cells, in response to the same dose of the chemotherapeutic agent, DOXO. The mechanism of DOXO-induced apoptosis has not been well classified but is reported to act by inhibiting topoisomerase II and generating hydrogen peroxide, which causes oxidative DNA damage [204]. Apoptosis was assessed by Western blotting, probing for cleaved PARP-1 using a specific antibody. PARP-1 is a caspase 3 substrate, such that levels of cleaved PARP-1 rise following the initiation of the apoptosis caspase cascade. I subsequently sought to investigate whether modulating GRK5 levels alters the sensitivity of cells to chemotherapeutic agents in the same cell line. GRK5 was overexpressed in HT-29 cells and two different assays were performed following treatment with the colon cancer drug, 5-FU: measuring cell viability by FACS and staining for apoptotic cells with an anti active caspase 3 antibody by immunofluorescence. In both experimental cases, GRK5 overexpression increases the percentage of apoptotic cells and this effect is exaggerated following treatment with the chemotherapeutic agent, 5-FU. Examining cell morphology following GRK5 overexpression and 5-FU treatment shows cells to adopt a more spherical shape (Figure 5.8), concomitant with apoptosis induction. Thus, apoptosis in HT-29 cells can be increased by GRK5

overexpression, suggesting that indeed GRK5 increases sensitivity to chemotherapeutic agents, potentially via the repression of Bcl-2.

Treatment with the class I HDAC inhibitor, MS-275, reduces 5-FU-mediated, GRK5-dependent apoptosis by approximately 50%, demonstrating that class I HDACs are important for the GRK5-mediated enhancement of apoptosis. Co-expression of GRK5 with the Sin3A mutant, N1015, reduces apoptosis by approximately 65%. This is the longest Sin3A deletion fragment, which encompasses the C-terminus, including the HID, PAH4 and the HCR and includes the GRK5 binding domain. I therefore hypothesise, as previously discussed, that this Sin3A fragment acts as a competitive inhibitor with endogenous Sin3A for GRK5, preventing GRK5 binding to the functional Sin3A complex and thereby inhibiting Sin3A-mediated effects of GRK5. This notion is supported by the observation that the GRK5-mediated transcriptional repression of Gal4 luciferase gene targets is slightly but significantly inhibited by the N1015 fragment and to a lesser extent the N680 fragment. The N1015 mutant also inhibits GRK5-mediated apoptosis but the N680 Sin3A mutant does not. As mentioned previously, although the HID is the most critical Sin3A domain for GRK5 binding, perhaps the remaining Sin3A C-terminal domains are necessary for maintaining the interaction with the kinase, potentially by recruiting other proteins to the complex to help stabilise the interaction. The apoptosis phenotype lies downstream of Bcl-2 regulation, such that the disruption of GRK5/Sin3A binding by N680 may not have been strong enough to affect GRK5-mediated, Sin3A-dependent apoptosis.

6.2.1 GRK5-dependent apoptosis occurs via p53 dependent and independent mechanisms

In osteosarcoma cells, GRK5 phosphorylates the tumour suppressor protein, p53, to inhibit p53 induced apoptosis [49], yet here we show GRK5 promotes apoptosis in HT-29 cells. P53 is a key player in the cellular response to stress and it has a well-established role in promoting apoptosis by transcriptionally regulating components of both the extrinsic and intrinsic apoptotic pathways [73]. These pathways of p53-dependent apoptosis, however, are not operative in all cancer cell lines; p53 is mutated in over 50% of cancers and over 75% of these mutations render the protein inactive in a manner that is dominant over any remaining wildtype protein [205]. Alternative, p53-independent apoptotic signalling pathways can be induced in such cases. Indeed, the cyclin-dependent kinase inhibitor, p21, is tightly regulated by p53, yet in HT-29 cells, which are p53 deficient, induction of p21 can occur in a p53-independent manner [206]. In HT-29 cells, the p53 gene carries a point missense mutation (Arg273His) in the DNA binding domain to render the protein inactive [207, 208]. I can, therefore, reconcile the opposite roles played by GRK5 in apoptosis regulation in osteosarcoma cells and in HT-29 cells, based on differences in p53 status. I propose that in HT-29 cells where p53 is non-functional, GRK5 enhances apoptosis sensitivity in a Sin3A dependent manner, via a novel mechanism that is distinct from and has opposing cellular consequences to that regulated by p53.

P53 is a molecular target of 5-FU and although its mutation accounts for apoptosis resistance, 5-FU can induce apoptosis in p53 deficient cell lines, albeit to a lesser extent. Indeed, as illustrated in Figure 5.8, GRK5-mediated apoptosis is increased

in HT-29 cells following 5-FU treatment. The mechanism of p53-independent apoptosis is not currently known but interferon-α has been shown to induce expression and activation of double stranded RNA (dsRNA)-dependent protein kinase (PKR), which is an innate immune and antiviral protein that plays a role in cell differentiation, growth and apoptosis. Stable knockdown of PKR reduces 5-FU induced apoptosis in the colon cancer cell line, HCT116, compared to control cells. Furthermore, this reduction in apoptosis is similar in HCT116 $p53^{+/+}$ and HCT116 $p53^{-/-}$ cells, thus suggesting that 5-FU is capable of inducing a p53-independent apoptosis mechanism via PKR [209]. Apoptosis was inferred from levels of Annexin V staining, which is a calcium-dependent phospholipid-binding protein that binds to phosphatidylserine to identify apoptotic cells. Apoptosis induction causes the relocalisation of phosphatidylserine from the cytosolic side of the plasma membrane to the extracellular matrix, where it is available for detection by fluorescently labeled Annexin V. Similar to GRK5, PKR is a serine/threonine kinase whose autophosphorylation is required for kinase activation. PKR phosphorylates translation initiation factor, eIF2α, inhibiting its activity as well as protein synthesis [210].

In a mouse xenograft model, colon cancer cells with reduced PKR expression established solid tumours faster than control cells, which were also resistant to DOXO treatment [211], implying that lower expression levels of PKR correlate with increased severity of colon tumours. Additionally, 5-FU treatment increases PKR protein expression levels in HCT116 cells following 24 hours treatment with 10 μM 5-FU [209]. In HT-29 cells, levels of GRK5 are low and overexpression of GRK5 renders HT-29 cells sensitive to 5-FU treatment, as shown in Figure 5.8 and Figure

5.10. It is therefore unlikely that 5-FU treatment increases GRK5 protein expression or activation, as overexpression of the kinase is necessary to induce sensitivity to 5-FU. Western blotting to detect GRK5 protein expression levels in 5-FU treated and untreated cells would, however, ascertain whether this is the case. Either way, GRK5 functions similarly to PKR to induce a p53-independent mechanism of apoptosis in response to 5-FU treatment in HT-29 cells.

6.2.2 GRK5 mediates apoptosis regulation

Data from my thesis points to a p53-independent mechanism of apoptosis induction by GRK5, potentially involving the Sin3A-mediated repression of Bcl-2 transcription. Apoptosis is a highly regulated cellular event and GRK5 is implicated in multiple signalling pathways that converge on apoptosis, suggesting that direct regulation of Bcl-2 levels may not be the only mechanism whereby GRK5 promotes apoptosis.

Bcl-2 is transcriptionally activated by NFκB, which itself is regulated by GRK5, as explained in detail in section 1.3.3.1 [65, 66, 69, 70, 212, 213]. DNase I footprinting analysis with purified NFκB isoform, p50, identified a putative binding site for the transcription factor within the Bcl-2 P2 promoter site. The ability of this promoter region to interact with nuclear components in prostate cancer cell line, LNCaP, was tested by gel retardation and supershift analysis and the NFκB heterodimer p50/65 was detected following probing with specific antibodies. The heterodimer activates Bcl-2 transcription, with overexpression of p50/65 in HeLa cells increasing transcription of a luciferase construct fused to the P2 promoter region of Bcl-2. Bcl-2 protein expression levels and transcription were increased following TNFα-stimulation and abrogated by preventing the phosphorylation of IκBα, either through

the use of inhibitors or a dominant negative IκBα construct [212]. Bcl-2 is upregulated in the early stages of colon cancer and NFκB is constitutively activated in a range of cancers, including colorectal [210]. Indeed, one such study found NFκB to be constitutively activated in 40% of tumours and 67% of cell lines, including HT-29 cells, albeit at relatively low levels compared to other colorectal carcinoma cell lines such as DLD-1 and LOVO. Knockdown of NFκB in colorectal cancer cell lines expressing high levels of NFκB, increased 5-FU induced apoptosis and reduced tumour expansion in mice by 44% compared to wildtype mice [210]. The concomitant down-regulation of GRK5 and upregulation of NFκB activity in colon cancer may therefore function to coordinately upregulate Bcl-2 expression. Furthermore, GRK5 is able to regulate NFκB, providing another avenue for Bcl-2 regulation. The functional consequence of GRK5-mediated NFκB regulation however, may depend on the stimulus, cell line and IκB member involved.

Following TNFα-stimulation in macrophages, GRK5 binds and phosphorylates the NFκB inhibitor, IκBα, leading to its degradation and subsequent NFκB activation. Nuclear translocation of NFκB ensues and its target genes are subsequently upregulated [70]. The same group, however, showed that GRK5 negatively regulates TLR4 induced ERK activation in macrophages by inhibiting the phosphorylation of NFκB subunit, p105 [66]. A similar, TLR4 induced, negative regulation of NFκB by GRK5 was reported in endothelial cells, this time via the stabilisation of the NFκB/IκBα complex [65]. The mechanism of GRK5-mediated NFκB activation is likely to be evolutionarily conserved in Drosophila, zebrafish and mice, resulting in upregulated cytokine expression [69, 71]. While NFκB is activated in colon cancer, based on the literature and from screening GRK5 protein

expression levels in a range of cancer cell lines (Figure 5.1), I hypothesise that GRK5 is downregulated in the diseased state. Based on these data, reduced expression of GRK5 may therefore relieve the NFκB inhibition and may account for the upregulated Bcl-2 expression.

The resultant phenotype of GRK5 overexpression in HT-29 cells is an increase in 5-FU induced apoptosis. NFκB has a well-known role in the immune response, particularly inflammation, but it also has pro- and anti-apoptotic functions depending on the inducing stimulus. TNF-α, ionizing radiation and the chemotherapeutic drug, daunorubicine, initiate an anti-apoptotic NFκB pathway. NFκB also inhibits p53-independent apoptosis induced by oncogenic Ras [214, 215]. The viability of Rat-1 cell lines stably co-expressing oncogenic Ras and IκBα was reduced by over 50% compared to control Rat-1 cells overexpressing oncogenic Ras alone. The same phenotype was observed in p53 null MEFs with combined overexpression of oncogenic Ras and IκBα, suggesting that inhibition of NFκB is having a pro-apoptotic effect [215]. These findings are in keeping with my hypothesis that in HT-29 cells, where p53 is mutated, NFκB is likely to function in an anti-apoptotic manner, in keeping with its role as an activator of Bcl-2 transcription. Overexpression of GRK5 may therefore serve to upregulate a 5-FU induced, p53-independent mechanism of apoptosis by promoting Sin3A-mediated and maybe NFκB-mediated inhibition of Bcl-2 transcription.

In addition to upregulating apoptosis regulatory pathways, another means of increasing susceptibility of cells to programmed cell death is by reducing proliferation. Stimulation of Wnt signalling converges upon β-catenin, which is re-

localised to the nucleus and activates the transcription of genes including c-Myc and cyclin D1, which promote proliferation. APC is part of a multiprotein complex that phosphorylates β-catenin, triggering its degradation by the proteasome. The down-regulation of APC is one of the earliest events in the progression of a colorectal adenoma to a carcinoma, which results in upregulated transcription of Wnt target genes due to β-catenin stabilisation and excessive cell proliferation, which is a hallmark of cancer cells [40]. GRK5, as well as GRK6, phosphorylate the Wnt signalling receptor, LRP6, and thereby activate β-catenin nuclear localisation [41]. The down-regulation of GRK5 and APC in colon cancer reduces and activates Wnt signalling respectively, thereby causing opposing Wnt signalling phenotypes. The upregulation of Wnt signalling is concomitant with cancer pathogenesis, such that I would therefore predict that the down-regulation of APC plays a dominant role in the Wnt pathway over the regulation by GRK5. The role of GRK5 is therefore likely to be concerned with activating apoptosis rather than inhibiting proliferation.

The GRK5-mediated inhibition of Bcl-2 transcription is consistent with the ability of the kinase to activate 5-FU-induced apoptosis, but I am yet to show that the two cellular events are connected. The simultaneous forced overexpression of Bcl-2 and GRK5 in HT-29 cells may show whether 5-FU induced apoptosis is dependent on the reduced Bcl-2 levels. The results, however, may not be as anticipated, as apoptosis is controlled by a complex regulatory network, such that compensatory mechanisms may be initiated.

6.2.3 GRK5 may potentially promote cell death via autophagy

In addition to apoptosis, another means of regulating cell death is by autophagy, otherwise known as type II programmed cell death. Autophagy is a cellular auto-digestion process by which proteins and old organelles are transported to lysosomes for degradation. The role of autophagy in colon cancer is paradoxical, having pro-survival and pro-death functions depending on whether cells are stressed or unstressed, respectively [216]. Autophagy can compensate for apoptosis to initiate death of colon cancer cells but it has also been shown to limit 5-FU induced apoptosis [217, 218]. HCT116 cells with mutated Bak or PUMA, and thus defective apoptosis, displayed similar sensitivities to 5-FU compared to wildtype HCT116 cells. While apoptosis was impaired, autophagy was increased in HCT116 $Bak^{-/-}$ and HCT116 $PUMA^{-/-}$ cells and cell death was reduced when autophagy was inhibited by treatment with the inhibitor, 3-Methyladenine [217]. These data therefore suggest that autophagy can act as an alternative to apoptosis to initiate cell death. Conversely, autophagy can also limit chemotherapeutically induced apoptosis, as 3-Methyladenine treatment significantly increases 5-FU induced apoptosis in HT-29 cells. The upregulated apoptosis observed following combined 3-Methyladenine and 5-FU treatment is concomitant with reduced expression of the anti-apoptotic protein, Bcl-xl, compared to 5-FU treatment alone [218]. It is possible that GRK5 may also be down-regulating Bcl-xl, as well as Bcl-2 expression, in HT-29 cells. I focussed exclusively on investigating whether the GRK5-mediated Bcl-2 repression in HT-29 cells affected apoptosis, but it is possible that Bcl-2 repression may also affect autophagy. Bcl-2 inhibits Beclin 1-dependent autophagy in HT-29 cells by blocking the formation of the Beclin 1/vacuolar sorting protein 34 autophagic complex by binding to Beclin 1. Vacuolar

sorting protein 34 is a phosphoinositide 3-kinase that plays a role in vesicle nucleation to induce autophagy [219]. It may therefore be possible that GRK5 is not only promoting apoptosis but also autophagy, potentially via Bcl-2 and/or Bcl-xl down-regulation, to mediate 5-FU induced HT-29 cell death. Expression of a GFP-tagged microtubule associated protein light chain 3 construct has been used as an indicator of autophagy. Prior to 5-FU treatment, transfection of the construct into HT-29 cells, with or without GRK5, will facilitate the investigation of whether indeed GRK5 promotes cell death via an autophagic mechanism.

6.3 GRK5 in disease

I have outlined a novel GRK5-dependent mechanism operative in colon cancer cells that increases chemotherapeutically induced apoptosis in a class I HDAC- and Sin3A-dependent manner and, as such, I hypothesise that GRK5 may play a nuclear role as an HDAC regulator in colon cancer. A microarray study of gene expression profiles of patients with colorectal liver metastases identified GRK5 as being significantly under expressed in higher-risk patients [Chung, J., 2013, personal communication]. Furthermore, a patent application published by Dr Stephen Gruber of the University of Michigan stated that a whole genome association study identified a SNP in the intron of GRK5 to be associated with a 35% increased risk of developing CRC [Patent publication number: WO2009046422 A2]. It is possible that this SNP affects GRK5 expression levels, possibly down-regulating GRK5 expression in keeping with the microarray data. Data from my thesis extends previous work that showed GRK5 to negatively regulate Bcl-2 transcription in SHSY5Y cells and extends this regulation to HT-29 cells. It is therefore feasible that GRK5 may be playing a causative role in colon

cancer progression to a metastatic state; down-regulation of the kinase may relieve Bcl-2 transcriptional inhibition and render cells resistant to pro-apoptotic stimuli, thus accelerating cancer cell growth.

A major limitation of my work is that all my experiments have been performed in cell lines or *in vitro*, which limits the inferences I can make as to whether this mechanism is operative in human colon cancer patients. The natural progression of this work is to move *in vivo* and study whether GRK5 can affect colon tumour shrinkage in nude mice infected with colon cancer cells, in response to chemotherapy. Through the use of HDAC inhibitors, overexpression and knockdown of the key proteins, one could also ascertain whether HDACs and Sin3A may be playing a role in disease pathogenesis. There are well-established and characterised mouse models of colon cancer, with mutated APC genes and mutated β-catenin signalling pathways. Whether the overexpression of GRK5 in these mice models can compensate for these mutations is worth investigating.

One disease where GRK5 is known to contribute to disease progression and which has been studied in depth *in vivo,* is pathological cardiac hypertrophy. GRKs 2, 3, 5 and 6 are found in the heart, with expression of GRK2 and GRK5 being the highest. As discussed in section 1.4.1, while both GRK2 and GRK5 are implicated in heart failure, the kinases have distinct cardiovascular functions, with upregulated GRK2 common to hypertension and upregulated GRK5 common to left ventricular overload disease. The role played by GRK5 in the heart has only been appreciated in recent years, while GRK2, well known for its role in β-adrenergic receptor phosphorylation, has long been known to play a causative role in heart failure [85,

86]. It was only in 2008 that work published by the Koch group explained the significance of upregulated GRK5 levels in CVD pathology. In comparison to littermate control mice and transgenic mice with cardiac specific overexpression of GRK2, GRK5 transgenic mice developed exaggerated pathological cardiac hypertrophy, premature heart failure and death following stress by TAC [81]. This pathological phenotype was not observed in transgenic mice overexpressing a GRK5ΔNLS mutant, thus giving a mechanistic insight into GRK5-mediated pathological cardiac hypertrophy. The Koch group investigated this mechanism in NRVM and found GRK5 to act as an HDAC5 kinase. HDAC5 phosphorylation induces its nuclear export, relieving the inhibition of MEF2 and thus resulting in the upregulation of pro-hypertrophic foetal cardiac genes [81]. Unlike HDAC5 and the remaining class II HDACs, which are anti-hypertrophic, class I HDACs are pro-hypertrophic, with class I HDAC inhibitors having cardioprotective effects in mice [129]. Data from my thesis suggests that GRK5 may be regulating class I HDACs, such that targeting GRK5 may also be a valuable therapeutic option. This is discussed further in section 6.3.1.

6.3.1 Targeting GRK5 as a therapeutic option

CRC is the second largest cause of cancer mortality in the United Kingdom. 40, 695 cases were diagnosed in 2010 alone, with 15,708 CRC related deaths reported in the same year [Cancer Research UK]. A major contributing factor to CRC mortality rates is the stage at which the disease is detected. Patients diagnosed with stage I cancers, which are confined to the colon, have up to approximately 87% more chance of survival five years post diagnosis compared to those diagnosed with metastatic CRCs, yet only 39% of patients present with stage I

tumours [112] [American Cancer Society, Cancer Facts and Figures 2012]. While stage I cancers are often cured by surgery, 5-FU treatment is the main chemotherapeutic used to treat stage II or III CRCs post surgery. Treatment of stage IV metastatic CRCs represents a more serious clinical problem, however, as these cancers are often resistant to chemotherapeutic agents.

6.3.1.1 Chemotherapy

Adjuvant chemotherapy is given post surgery to stage III colon cancers, but its use in stage II tumours remains controversial, as survival rates following surgery alone are 75–80% [115]. HT-29 cells are derived from a colorectal adenocarcinoma and form well differentiated grade I or II tumours. Such tumours are most often operable but the risk of recurrence is high in some stage II tumour patients, particularly in advanced stages where the tumour has invaded neighbouring organs. In such cases, patients would most likely benefit from chemotherapy to reduce their long-term risk. HT-29 cells are widely used to investigate colon cancer therapeutic options and numerous studies of 5-FU resistance and toxicity have been carried out in these cells. Here, I have shown that GRK5 overexpression increases sensitivity of HT-29 to 5-FU in a class I HDAC and Sin3A dependent manner and that treatment alone does not promote apoptosis. These findings may therefore prove beneficial in advancing colon cancer treatment options. While increasing GRK5 expression levels in colon cancer cells of patients in order to boost chemotherapy efficacy is not practical, the use of miRNA strategies may provide a safe and efficacious method to achieve such results, while circumventing the need for chemotherapy. This is discussed in more detail in section 6.3.1.4.

6.3.1.2 HDAC inhibitors

Many studies have investigated the use of HDAC inhibitors to treat pathological cardiac hypertrophy, whose action is cardioprotective. Treatment of mice with pan-HDAC inhibitors such as TSA and NaB prevent cardiac remodelling, activation of the foetal cardiac gene program and improve cardiac function [125, 220]. These results seem paradoxical with research published by the Koch group suggesting that class II HDACs are anti-hypertrophic. The overexpression of class I HDACs 1, 2 and 3 are directly involved in cardiac myocyte proliferation and hypertrophy, thus implying that the two classes of classical HDACs have opposing functions in the context of this CVD [164, 221, 222]. Treatment with class I specific HDAC inhibitors has cardioprotective effects; following hypertrophic induction, disease development was significantly reduced in mice treated with the class I specific HDAC inhibitor, SK-7041 [129]. Whether the regulation of class I HDACs involves GRK5 is yet to be confirmed, but considering both proteins have pro-hypertrophic functions, they may well be involved in the same pro-hypertrophic pathway. If the GRK5/HDAC1/Sin3A complex is operative in pathological cardiac hypertrophy, I would expect the repressor complex to be involved in switching off adult cardiac genes. If the kinase activity of GRK5 is necessary to mediate this repression, potentially by activating HDAC1, small molecule inhibitors that disrupt the complex may be an effective treatment option.

HDAC inhibitors including NaB and MS-275 promote growth arrest, differentiation and apoptosis in colon cancer cell lines and *in vivo* [223]. Expression levels of class I HDACs are often upregulated in colon cancers, with upregulation of HDACs 1, 2 and 8 demonstrated at both protein and mRNA levels [223]. APC represses

HDAC2 in HT-29 cells, most likely at the transcriptional level, and knockdown of HDAC2 reduces cell growth [224]. HDAC gene expression is induced following loss of APC, leading to overexpression of the HDAC in the diseased state. In contrast to these data, the stable overexpression of HDAC2 in the colon cancer cell line, RKO, reduces proliferation, suggesting potentially cell-line dependent roles of the HDAC [225]. Class I HDAC upregulation in colon cancer is mechanistically linked to the repression of p21, which induces growth arrest [226]. HDAC inhibitors also promote apoptosis in colon cancer cell lines, with NaB increasing the expression of the pro-apoptotic protein, Bak. Furthermore, overexpression of Bcl-2 only conferred protection against apoptosis when Bak levels were not elevated. This suggests that the HDAC inhibitors exert their effects predominantly through elevating Bak levels as opposed to repressing Bcl-2 [227]. This is in keeping with my findings that NaB and MS-275 treatment do not affect Bcl-2 protein levels in HT-29 cells (Figure 5.3). Furthermore, treatment of HT-29 cells with MS-275 does not affect 5-FU induced apoptosis but the simultaneous overexpression of GRK5 and treatment with MS-275 reduced apoptosis, as observed by reduced cleaved caspase 3 staining (Figure 5.9). Overexpression of GRK5 represses Bcl-2 and thereby increases apoptosis in response to 5-FU in a class I HDAC-dependent manner. The overexpression of GRK5 and the induction of HDAC/Sin3A-mediated repression of Bcl-2 is likely to dominate over the possible upregulation of Bak induced by MS-275. So while HDAC inhibitors have protective effects in colon cancer, I propose that targeting the GRK5 mechanism of Bcl-2 repression may be more beneficial. As previously mentioned, the emerging use of miRNAs as therapeutics could prove to be a viable and efficacious method of boosting GRK5 expression levels and thus promoting GRK5-mediated apoptosis via Bcl-2 repression.

6.3.1.3 GRK5-NT

Constitutive activation of NFκB, which is common to many cancers including those in the colon, contributes to chemo-resistance [228, 229]. Regulating NFκB signalling may therefore provide an alternative means of increasing sensitivity to apoptosis and the Iaccarino group have investigated the role of GRK5 in this potential novel therapeutic avenue. Following the mapping of the NFκB binding site to the RH domain in the N-terminus of GRK5, use of a GRK5 N-terminal fragment (GRK5-NT) reduces NFκB activation and functions in a protective manner in cancer and hypertrophy [65, 93, 230] as described below.

An AdGRK5-NT construct reduced NFκB transcriptional activity in cardiomyoblasts following phenylephrine treatment, as a means of inducing hypertrophy. Moreover, *in vivo* studies showed that intracardiac injection of the construct inhibits hypertrophy development in spontaneously hypertensive rats and Wistar Kyoto control rats treated with chronic injection of phenylephrine [93]. In the human thyroid tumour cell line, KAT-4, overexpression of AdGRK5-NT blocked NFκB transcriptional activity and increased apoptosis. In BALB/c mice with KAT-4 induced neoplasias, intra-tumour delivery of AdGRK5-NT reduced tumour growth in a dose-dependent manner. Moving towards a more pharmacological method of GRK5-mediated inhibition of NFκB, the GRK5 N-terminal RH domain was fused to a synthetic TAT protein (TAT-RH) for active transport into cells, and tested for its anti-cancer properties. In KAT-4 cells and in BALB/c mice, TAT-RH reduced cell survival and tumour growth respectively. IκBα and cleaved caspase 3 levels were also increased in cells and mice [230]. TAT-tagged proteins can be effectively

transduced into human cells in tissue culture, although their expression is short lived [231].

6.3.1.4 miRNAs

170 different miRNAs have been reported to be upregulated in human CRC, including miR-135a and miR-135b [232]. Upregulation of these miRNAs correlate with reduced APC mRNA expression levels, which occurs early in the development of colon cancer [185]. Mutated APC and upregulated Wnt signalling promotes tumourigenesis by stabilising β-catenin [40]. miR-135a and b are predicted to target GRK5 in GBMs [188]. I have shown in Figure 5.10 that overexpression of an antisense miR-135a oligonucleotide upregulates GRK5 levels in HT-29 cells, which corresponds with increased apoptosis, as assessed by immunofluorescence. Overexpression of an antisense miR-135a or miR-135b oligonucleotide will undoubtedly increase APC expression levels, which will result in degradation of β-catenin. In addition to upregulating genes that control proliferation, β-catenin also upregulates Bcl-2 mRNA in HEK293 cells and in rats treated with heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine to induce colon cancer. Bcl-2 mRNA levels are upregulated in colon tumours containing mutations in the *CTNNB1* gene that encodes β-catenin [233]. The increased apoptosis observed following the inhibition of miR-135a may, therefore, be due not only to the upregulation of GRK5 and the concomitant Sin3A and HDAC1-dependent Bcl-2 repression, but also to upregulated APC expression. Increasing APC expression results in β-catenin degradation, which thereby reduces β-catenin-mediated Bcl-2 transcription.

The use of miRNA technology to treat colon cancer is already being explored. miR-143 is downregulated in the disease and a synthetic miR-143 mimic has significant tumour suppressive effects in mouse CRC models [234]. Much research is still needed to ensure the safe delivery of miRNA mimics and inhibitors and confirm their protective effects in patients. Research, however, is progressing, as the use of a miR-122 inhibitor is already being tested in a human phase II, hepatitis C virus clinical trial [182]. miR-122 is also upregulated in high-grade prostate tumours, such that that the development of an miR-122 inhibitor may be a useful cancer therapeutic [187]. A miR-135b inhibitor is already being investigated to treat lymphoma and nanoparticle delivery of the inhibitor reduced tumour growth in *in vivo* mouse models [235]. This method of delivering the miR-135b inhibitor has not been tested in colon cancer mouse models, but is definitely worth investigating.

6.4 Future work

6.4.1 Mapping the binding sites of HDAC1 and Sin3A on GRK5

Sin3A regulates transcription of a multitude of genes. Previous work has shown that mutating binding partners, such as the Sap30L protein, is an effective strategy to investigate their function, particularly if they are involved in a multiprotein repressor complex like Sin3A. Following the identification of Sap30 and Sap30L as Sin3A interactors, the binding site on Sap30L was mapped to the C-terminus. Transfection of a C-terminal truncated Sap30L mutant prevented Sin3A nuclear localisation that occurs following transfection with Sap30L wildtype protein [236]. Sin3A itself does not contain an NLS and these studies therefore highlight the role of Sap30L in localising Sin3A to the nucleus. With similar principles in mind, I

attempted to map the binding sites of HDAC1 and Sin3A on GRK5, in order to elucidate the function of these interactions.

Using GRK5 peptide arrays comprising 25mer immobilised peptides, I attempted to identify specific GRK5 peptides that mediate binding to HDAC1 and Sin3A. The arrays were incubated with the respective fusion proteins and developed as per a Western blot. Following the analysis of arrays developed from multiple experiments, specific peptides were selected based on repeated binding patterns to the target proteins; peptides 69 and 112 were selected as HDAC1 binding peptides, peptides 76 and 106 as Sin3A binding peptides and peptides 82 and 96 were detected in both HDAC1 and Sin3A experiments. To further investigate the potential binding sites, these peptides were incorporated into an alanine scanning substitution array. Analysis of the alanine scanning arrays highlighted three regions of GRK5 to mutate to potentially create GRK5ΔHDAC1, GRK5ΔSin3A and GRK5ΔHDAC1 ΔSIn3A binding deficient mutants. The following GRK5 mutants, M1–M5, were made: E355A/V356A, D485A, D486A, D487A and D485/486/487A (Table 4.2). The ability of these mutants to bind HDAC1 and Sin3A were examined in direct binding assays with immobilised GST-HDAC1 and GST-Sin3A-545-1157 and *in vitro* translated radiolabelled wildtype and mutant GRK5 constructs. None of the mutations made disrupted binding to the fusion proteins as compared to wildtype GRK5. Mutant M4, D487A, displayed enhanced binding capabilities to both HDAC1 and Sin3A, suggesting the final aspartate residue in this sequence is a negative determinant for binding to both target proteins. This result apparently contradicts what was observed in the alanine scanning array, whereby mutation of this final aspartate behaved like the ones previous and appeared to abolish binding to

HDAC1 and Sin3A. It could be argued that when in the tertiary complex, the mutation of this aspartate residue may alter protein folding and form a GRK5 mutant that is more receptive to interacting with HDAC1 and Sin3A.

A potential Sin3A binding site on GRK5 was identified between Arg388 and Lys389. A pre-existing GRK5ΔNLS mutant in which residues Arg388, Lys389, Lys391, Lys393 and Arg394 are mutated to alanine has already been tested and characterised, such that I decided to test this construct as a potential binding mutant. Indeed, binding of the GRK5ΔNLS to both HDAC1 and Sin3A was inhibited; *in vitro* translation of GRK5ΔNLS and incubation with both GST-HDAC1 and GST-Sin3A-545-1157 resulted in weaker binding to the fusion proteins in comparison to wildtype GRK5. The caveat of using this pre-existing GRK5ΔNLS mutant, however, is that any differences in binding cannot be attributed to residues Arg388 and Lys389 alone, as additional residues are mutated to exclude GRK5 from the nucleus. So despite GRK5ΔNLS functioning as a GRK5ΔHDAC1 and GRK5ΔSin3A binding deficient mutant, I cannot use it to further investigate the physiological implications of the GRK5/HDAC1 and GRK5/Sin3A interactions in a cellular setting; any notable alteration in phenotype cannot be attributed solely to perturbed binding to HDAC1 and Sin3A but instead may be due to the exclusion of the kinase from the nucleus.

Many of the spots chosen from the peptide arrays for alanine scanning mutation were single peptides that appeared repeatedly on the arrays in multiple experiments, such that I presumed these peptides were important for mediating binding. These include peptides 69, 76, 96 and 82. In hindsight, selecting peptides

that were part of a run of positive interactors may have been more prudent. A string of dark spots can been seen on the array in Figure 4.3 probed with GST-Sin3A-545-1157, running inclusively from peptides 106–112. Considering the peptides overlap by five amino acids, it is most likely that more than one peptide will mediate a binding event. Choosing a peptide included in a run of positively interacting spots rather than individual, isolated spots may have been wiser. That being said, the Baillie group successfully mapped the binding site of PDE4D5 on RACK1 by selecting one isolated peptide from RACK1 peptide arrays incubated with full-length PDE4D5 [237].

In light of these array results I investigated a functional role for nuclear GRK5 in coordination with HDAC1 and the Sin3A complex and studied the importance of this interaction through the use of HDAC and Sin3A inhibitors. While this worked effectively to illustrate the Sin3A and class I HDAC dependence of GRK5-mediated Bcl-2 repression and apoptosis, It is worth revisiting the arrays in the future, to repeat the alanine scanning arrays with different GRK5 peptides and potentially create GRK5ΔHDAC1 and GRK5ΔSin3A binding deficient mutants.

6.4.2 GRK5 is a potentiator of apoptosis

Data from my thesis suggests GRK5 is a potentiator of apoptosis, which could have considerable therapeutic implications. In order to pursue this hypothesis it is imperative to test whether my findings hold true *in vivo*. Microinjection of human colon cancer cell lines, SW620, HCT116 and DLD-1, into the colonic mucosa of nude mice cause colon tumours to development in up to 88% of infected mice [238]. By comparison, injecting colon cancer cells overexpressing GRK5 into nude mice

may halt or delay tumour growth and is a study worth performing. Before commencing with *in vivo* studies, however, it may be interesting to repeat the apoptosis experiments in SW620 cells, or another metastatic colon cancer cell line. HT-29 cells are models of early stage colon cancer, of grade I or II tumours. Many patients present with later stage colon cancers, which are more akin to the SW620 cell model. While the results I have obtained in HT-29 cells are useful in understanding the regulation of apoptosis in colon cancer, it would be advantageous to know whether similar mechanisms are still functional later in disease pathology.

If GRK5 is indeed downregulated in colon cancer, as hypothesised, this would highlight the potential use of GRK5 as a disease biomarker. While I have shown GRK5 expression levels to be lowest in HT-29 and SW620 cells in comparison to other tumour derived cell lines, this does not confirm whether indeed GRK5 is downregulated in the disease. In order to pursue this investigation it would be advantageous to test GRK5 expression levels in human subjects with the GRK5 SNP that increases the risk of CRC development by up to 35% (Patent publication number: WO2009046422 A2). Following an endoscopic biopsy to collect a sample of colon cells, GRK5 mRNA levels could be tested by RT-PCR experiments and compared to cells from control subjects without the GRK5 SNP. Comparing GRK5 expression levels between CRC and control patients would ascertain whether indeed GRK5 levels are reduced in the disease. Furthermore, if a difference in GRK5 levels is observed between subjects with and without the GRK5 SNP, this may suggest that down-regulation of GRK5 is an early event in CRC development, possibly predisposing subjects to the disease. It is estimated that 60% of CRC

related deaths could be prevented if screening methods were more widely utilised [113]. If reductions in GRK5 expression levels occur in the early stages of disease pathology then screening for kinase levels may represent a novel diagnostic approach.

An initial experiment suggests that induction of apoptosis by GRK5 may not be limited to colon cancer. OVCAR3 cells, which have 10-fold higher expression levels of GRK5, were more sensitive to DOXO treatment compared to HT-29 cells (Figure 5.7). It will be interesting to note whether GRK5 levels correlate with apoptosis sensitivity in a range of cancer cells and whether GRK5 can halt the growth of different tumours *in vivo*. Like HT-29 cells, OVCAR3 have mutated p53. Comparing apoptosis in cancer cells with wildtype and mutated p53 will ascertain how important functional p53 is in mediating apoptosis and whether the mechanism of GRK5-induced apoptosis, via transcriptional repression of Bcl-2, also functions in cells with functional p53.

It will also be interesting to note whether the mechanism of GRK5-mediated transcriptional regulation, shown here in HT-29 cells, is also functional in pathological hypertrophic hearts. GRK5 is upregulated in patients with left ventricular overload disease, which is associated with hypertrophy and nuclear localisation of the kinase is important for regulation of HDAC5 [81, 87]. GRK5 may, therefore, also be functioning to repress Bcl-2 in hypertrophic cardiac myocytes, as a pathological hallmark of the disease is increased myocyte apoptosis [239]. Indeed, chronic pressure overload in rat hearts causes significant apoptosis, which correlates with reduced expression of Bcl-2 [240].

6.4.3 Other GRK4 subfamily members

Like GRK5, GRK4 and GRK6 also have functional NLSs and adopt a nuclear localisation in HEp2 cells [11], suggesting that these GRKs also have the potential to be involved in regulating transcription. The NLS of GRK4, unlike GRK5 and GRK6, is unable to interact with DNA [10], such the mechanism shown here for the role of nuclear GRK5 as a transcriptional regulator and inducer of apoptosis, may be applicable to GRK6 but perhaps not GRK4. Investigating whether GRK4 can affect the sensitivity of HT-29 cells to apoptosis via Sin3A and HDAC1 may shed further light on the GRK5-mediated mechanism of gene repression, possibly indicating whether DNA binding is important. Performing co-immunoprecipitation experiments in HeLa cells co-expressing Sin3A or HDAC1, as well as either GRK4 or GRK6, would test whether these kinases, like GRK5, bind HDAC1 and Sin3A in cell lysates. Testing the ability of GRK4 and GRK6 to bind to all the HDACs would identify whether the specificity of GRK5 interacting with class I over class II HDACs is GRK specific. While the mechanism of GRK5-mediated regulation of HDAC/Sin3A-controlled transcription may be operative in cardiac myocytes to repress adult cardiac genes, it is unlikely that GRK4 or GRK6 will function in a similar manner, as GRK5 and GRK2 are the predominant GRKs expressed in the heart. This, however, does not rule out the possibility that a potential mechanism of GRK4 or GRK6-mediated gene repression may be operative in other cell types, including colon cancer, although no known nuclear substrates or binding partners of either kinase have currently been reported. While GRK5 binds to Sin3A, perhaps GRK4 and GRK6 bind to other repressor complexes. Additionally, extending these investigations to include the GRK2 subfamily would also be of interest.

6.5 Concluding remarks

In conclusion, I present data in this thesis highlighting a role for GRK5 as a transcriptional repressor, repressing Bcl-2 gene expression and increasing chemotherapeutically induced apoptosis in HT-29 cells. Since the discovery of the GRK4 subfamily NLS and NES sequences in 2004, numerous nuclear substrates and binding partners of GRK5 have been identified, expanding the repertoire of GRK5 functions far beyond GPCR regulation. GRK5 is implicated in a variety of diseases, including pathological cardiac hypertrophy and cancer. GRK5-mediated pathological cardiac hypertrophy depends on the nuclear localisation of the kinase and the data presented in this thesis suggests the same is true for GRK5-mediated colon cancer pathogenesis.

Here, I have shown for the first time, a potential mechanism of GRK5-mediated gene repression by acting as a component of the Sin3A transcriptional repressor complex. Through directly binding Sin3A and HDAC1, I infer that GRK5 is able to repress Bcl-2 transcription and thereby increases apoptosis induced by DNA-damaging agents. In the colon cancer cell line, HT-29, where levels of GRK5 are low in relation to cells derived from other tumours, GRK5 overexpression by the method of cDNA transfection, increases apoptosis in response to 5-FU treatment. Moreover, overexpression of a miR-135a inhibitor enhances endogenous GRK5 protein expression as well as apoptosis in untreated HT-29 cells, thus representing a potentially valuable therapeutic strategy in the treatment of colon cancer. Provisional experiments suggest that apoptosis regulation by GRK5 may not be limited to colon cancer, but may represent a universal function of the kinase; OVCAR3 cells, which have higher endogenous GRK5 expression levels, are more

sensitive to DOXO treatment. While these findings need to be confirmed *in vivo*, my work highlights the potential for a GRK5-targetted therapy as a means of treating colon cancer patients, for which the use of a miR-135a inhibitor may be effective.

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